University of Alberta

Chemosensory Cues of Larviposition Behavior of the Flesh Fly, *Neobellieria bullata* (Parker) (Diptera: Sarcophagidae)

by

Gregory Dennis William Pommen ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Entomology

Edmonton, Alberta

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Greg Pommen

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Date: June 10, 1996

"...that in nothing elswhere is more seen the workmanship of Nature, than in the artificiall composition of these little bodies... how can one comprehend the reason, the power, and the inexplicable perfection that Nature hath therin shewed?... How hath she bestowed all the five senses in a Gnat? ... where hath she made the seat of her eies to see before it? where hath she set and disposed the tast? where hath she placed and inserted the intrument and organ of smelling?"

"... I would request therfore the Readers, that in perusing this treatise, they will not come with a prejudicate opinion, nor (because many of these silly flies and wormes be contemptible in their eies) disdaine, loath, and contemne the reports that I shall make thereof; seeing there is nothing either in Natures workes that may seeme superfluous, or in her order unworthy our speculation."

- Natural History by Pliny the Elder (translated by Philemon Holland)

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Chemosensory Cues of Larviposition Behavior of the Flesh Fly, *Neobellieria bullata* (Parker) (Diptera: Sarcophagidae)** submitted by **Gregory Dennis William Pommen** in partial fulfillment of the requirements for the degree of **Master of Science**.

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Date: May 28, 1996

Dedication

I dedicate this to Fred and Betty Clark who were not able to see the completion of this project.

Abstract

Larviposition behavior of the flesh fly, *Neobellieria bullata* (Diptera: Sarcophagidae), was examined focusing on the chemically mediated aspects. Larviposition responses of females, number of larvae deposited, age of larvipositing females, the link between taste and larviposition, and mechanisms controlling larviposition were examined using data collected from observations of individuals, dissections, and rearings. Chemical analysis of Sigma's liver concentrate was performed using SDS-PAGE, capillary gel electrophoresis, micellar electrokinetic capillary chromatography, and HPLC to examine protein, nucleotide and amino acid content of a larviposition substrate. These analyses also demonstrated that chloroform: methanol: water extraction is a specific method for removal of lipid components. Without lipid constituents, liver is a less effective larviposition stimulant; however some individuals do not require lipid for normal larviposition behavior. High variation in responses are proposed to be extremely important in allowing populations of flies to survive a highly variable and competitive environment.

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Chapter 1 Introduction to Thesis

1.1 Chemically Mediated Behavior: Introduction

Chemically mediated behaviors are probably the oldest among organisms since even single celled organisms can react to the presence of chemical compounds. In more complex organisms, such as arthropods, molluscs, and vertebrates, specific morphological and behavioral adaptations have developed to take advantage of chemical information. These animals have developed complex peripheral sensory structures and integrative centers in the central nervous system to coordinate a diverse array of behavioral activities as a result of the perception of important chemical compounds.

Chemically mediated behavior encompasses many levels of organization, from receptor molecules on chemosensory neurons to ecological aspects of behavior. The work presented in this thesis focuses primarily on behavior of individual animals in this case a flesh fly, *Neobellieria bullata* (Parker).

1.2 Trends in Research on Chemically Mediated Behavior of Flies

Behavioral research over the past 40 years has attempted to relate the actions of chemosensory neurons with the behavior they mediate, otherwise known as the "top-down" or "bottom-up" approaches (Heiligenberg, 1991). Calyptrate flies have been commonly used for this type of research, especially species from the families Calliphoridae, blow flies, and Sarcophagidae, flesh flies. These flies are generalists and respond to a wide variety of chemical stimuli, both simple and complex, for feeding and oviposition behavior. Another useful aspect of flies is the structure of their contact chemoreceptors or taste hairs. Analogous to a vertebrate taste bud, these hairs consist of four chemosensory neurons plus a mechanoreceptor into a long, isolated hair. With the development of the tip recording technique of Hodgson et. al. (1955) and the side-wall technique of Morita (1959) it became possible to record the activity of chemosensory neurons to different stimuli. Integrated studies of chemoreception and chemically mediated behavior became possible.

The integrated approach has been highly useful but it has some notable limitations. Chemical compounds tested must have significance to the flies but must also satisfy the limitations of current neurophysiological techniques. Due in part to the influence of neurophysiological techniques, chemical compounds of choice are usually low molecular weight, water soluble, and nonvolatile in nature. The use of salts, sugars, and amino acids in this type of research is common with occasional use of short chain fatty acids, peptides, proteins, and more complex substrates such as liver, fish meal, beer, and apple mixtures (Albert et.al., 1991; Dethier, 1961; Dethier and Hanson, 1968; Dethier, 1974; Evans, 1961; Hanson, 1987; Jakinovich jr. et. al., 1971; Maes and Bijpost, 1979; Maes and Harms, 1986; Maes and Ruifrok, 1986; Maes and Vedder, 1978; Mitchell et.al., 1990; Ninomiya and Shimada, 1976; Shimada, 1975a; Shimada, 1975b; Shimada, 1978; Shimada, 1987; Shimada and Isono, 1978 Shimada and Tanimura, 1981; Shimada et.al., 1983; Shimada et.al., 1985; Shimada et.al., 1989; Shiraishi and Hiromichi, 1969; Shiraishi and Kuwabara, 1970, van der Molen et. al., 1985). The simpler substrates satisfy the demands of neural recording and also elicit behaviorally significant feeding responses. However, with more complex chemical substrates neurophysiological techniques are less useful. In addition, olfactory stimuli can not be accounted for which are especially important for oviposition behavior.

1.3 Oviposition/Larviposition

Oviposition is a complex behavior and usually involves complex substrates; living animals, carrion, or excrement. Most species require complex interactions of olfaction, taste, and other cues such as tactile stimuli for attraction and stimulation; hence oviposition and larviposition are still not understood in great detail. Despite superficial similarities in morphology and habits much variability exists among species and even within species.

1.3.1 Chemical Stimuli

With respect to chemical stimuli of oviposition, the most well studied flies are sheep blow flies, *Phaenicia sericata* and *Lucilia cuprina*, two major causes of dermal myiasis. In these species certain volatile compounds may attract but do not necessarily stimulate oviposition. Other compounds play roles in both attraction and stimulation. While various simple chemical compounds are involved in oviposition none of these will stimulate flies when offered alone; all require the addition of an unknown "wool" or "sheep" factor. Carbon dioxide is considered an important attractant and oviposition stimulant for both species, Cragg (1950; 1956) and Cragg and Thurston (1950) also demonstrated that *P. sericata* requires ammonia. They also suggested sulphur compounds, especially hydrogen sulphide, and indole are important for *P. sericata* as stimulants and/or attractants. However, Easton and Feir (1991) were not able to confirm that indole is an effective attractant for *P. sericata* . Emmens and Murray (1982) suggested sulphur compounds from bacteria were important odors for *L. cuprina* .

Stimulants for carrion feeders are less well known and in some cases the evidence is conflicting. Wardle (1921) wrote a summary of some compounds suspected to attract and/or stimulate flies to oviposit on meat in stores. He suggested that proteins are important, possibly albumins or globulins, although this was not confirmed. Cooking meat prevented flies from ovipositing. Putrefication bases such as ammonia, dimethylamine, guanine, indole, methylamine, methylguanidine, putrescine, skatole, trimethylamine, and tyrosine were also suggested as stimulants. For the most part these compounds have never been demonstrated to be effective stimulants, and as Wardle (1921) pointed out, carrion feeding flies will deposit on fresh animal flesh where these bases are not present in large quantities.

Only one reference exists for a specific compound inducing larviposition when acting alone. Howlett (1912) claimed to have "...observed a species of *Sarcophaga* to deposit larvae in a flask containing a solution of skatol, a compound present in the feces of many animals.". No other information is given regarding experimental conditions or even which species of *Sarcophaga* was observed. No other authors have reported a successful reproduction of this experiment. Easton and Feir (1991) suggest that skatol may even act as a repellent to *P. sericata*, and this is not surprising considering this fly attacks live animals.

Sarcophagids will larviposit on a wide range of substrates with diverse chemical composition. *N. bullata* alone has been collected from a wide range of substrates including fresh and decaying meat, vertebrates and invertebrates, mammalian excrement, and living mammals as well (Graenicher, 1931; Graenicher, 1935; James, 1947; Kamal, 1958; Knipling, 1936; Knipling and Rainwater, 1937). Chemical stimuli associated with larviposition are likely to be very general to encompass this diversity.

1.3.2 Taste versus Olfaction

Chemical compounds are perceived using the senses of taste and olfaction while physical properties are sensed as tactile stimulation. The integration of these sensations in the central nervous systemproduces a sensation representative of a particular oviposition substrate to a fly. With vertebrates we refer to this integrated sensation as flavor, which is a result of the combination of taste, olfactory, and tactile stimuli (Hornung and Enns, 1984). Given its distinctly anthropomorphic connotations, the term flavor may leave a bitter taste in the mouths of some ethologists, but it may apply equally well to flies.

The relative roles played in oviposition by olfaction and taste differ from species to species and may even differ within a species depending on external and internal conditions. *Phormia regina* appears to place great emphasis on olfactory information and will oviposit almost solely in response to the odor of an oviposition medium (Barton Browne, 1960). Olfactory stimuli are also the primary chemical sense for *Lucilia cuprina* but in this case tarsal contact with water is required (Barton Browne, 1962), indicating a need for contact chemoreception. Olfaction is important to the primary screwworm, *Cochliomyia hominivorax* , as well but taste plays an important role in modifying the response as does tactile stimulation (Hammack, 1991).

Far more uncertainty is present in the literature regarding the subject of this study, *N. bullata* . Mitchell and Soucie (1993) report this species will not larviposit without first tasting a suitable substrate using their labellum. Furthermore olfaction may serve to induce a central nervous system excitatory state (CES) when the fly is placed in the presence of odors from a suitable substrate. This is the first time rigid experimental procedures have been used to look at the relative roles of olfaction versus taste in a member of the Sarcophagidae and the results stand in contrast to those of earlier work. As early as 1921, Wardle believed sarcophagids in general oviposited in response to olfactory cues whereas calliphorids deposited chiefly in response to gustatory stimuli. Graenicher (1935) observed *Sarcophaga bullata* dropping larvae through cheesecloth covered bottles in response to the odors of decaying beetles. Sarcophagid larvae including *N. bullata* have been recovered from covered, baited containers by Graenicher (1935) and Hallock (1942). This led James (1947) to speculate that misidentification of myiasis causing species could result from sarcophagids depositing into covered jars containing human stool specimens. There are clear discrepencies in the literature regarding this species, possibly caused by: experimental error, misindentification of species, variability between populations, different experimental conditions between studies, or variability in larviposition behavior.

1.3.3 Variability

Variability in larviposition is commonly accepted in the literature; however, little quantification has been performed. This is not surprising, since larviposition behavior is normally studied by observing groups of flies rather than individuals. The only recent description of larviposition behavior comes from Mitchell and Soucie (1993), whose observations indicated that high variation was present between individuals.

Clearly this variation needs to be described in furthur detail as variability is an important part of behavior. Not only is this information needed for designing experiments but several researchers have described potential consequences of variation in responses of flies. Denlinger and Zdarek (1994) report that *N. bullata* pre-pupae ranging from 20 mg to 120 mg may successfully emerge as adults. Pappas and Fraenkel (1977) report *N. bullata* adults will develop viable eggs from pupae with weights from 20 to 130 mg. Plasticity is likely an important adaptation for animals that develop on limited and patchy resources (Denlinger and Zdárek, 1994). Denlinger (1978; 1979) showed that a few rare individuals of tropical sarcophagids diapause when conditions do not warrant. He furthur suggested these few "outlier" individuals may play an important role as genetic "fail-safes" for survival under occasional extreme conditions. Another carrion-feeding blowfly, *Chrysomya megacephala* , commonly attacks drying fish in open air markets of southeast Asia, Australia, and recently Africa. Salt is a common method of preventing attack, but this fly exhibits high degrees of variability in larval survival and adult preferences. It may deposit on and in some cases survive on fish that is 40% salt by dry weight (Esser, 1990). Variance of chemoreceptor cell responses has been proposed as a mechanism of coding at the peripheral chemosensory level for *N. bullata* (Mitchell et. al., 1990). Van der Molen, et. al. (1985) demonstrated the importance of quantifying several aspects of variation in blow fly taste responses for neurophysiological studies of chemoreception.

1.4 Objectives

Larviposition behavior of the species, *N. bullata*, was explored in this study, with chemosensory stimuli the primary focus. This was accomplished by: 1) quantifying the variability of larviposition behavior and where possible other aspects of the animal's biology 2) examining the role of olfaction and taste as mediators of larviposition 3) performing a brief analysis of the chemical makeup of a known larviposition substrate and 4) using bioassays to evaluate a derivative of a larviposition substrate.

The variability of various aspects of the biology of this fly was examined with a focus on larviposition behavior. The work required the examination of chemosensory stimuli mediating behavior and yielded a plethora of data regarding various aspects of larviposition, most of which have never been described before. The process of rearing animals, various dissections, and other work also yielded information about various aspects of the animal's biology. These data are considered relevant to explaining the results presented in this work and for designing future studies.

The role of olfactory input on larviposition was examined. A bioassay was designed to determine if exposure to odors of a substrate could: 1) stimulate larviposition and 2) generate a central nervous system excitatory state (CES) or "precondition" flies to larviposit more quickly upon arrival at a substrate. From this experiment, the role of taste in larviposition was investigated.

Physical and chemical properties of a larviposition substrate were evaluated. A known larviposition substrate, a liver derivative, was subjected to a chloroform: methanol: water extraction. Three products resulted and were quantified: a water soluble fraction, a lipid fraction, and insoluble material. Quantitative analysis of the liver derivative and water soluble fraction were performed using HPLC, capillary electrophoresis, and slab gel electrophoresis to examine if major chemical changes resulted from the extraction procedure. Although analysis of meat products is common in the food industry, such studies ignore the concerns of flies and prefer to concentrate on the nutritional and taste significance to humans. Results of a brief analysis of the liver derivative will be presented here focusing on amino acid, nucleotide, and protein content.

Stimulus strength of the water soluble fraction versus the original derivative was compared using behavioral bioassays. This method was used to determine if water soluble components from a larviposition substrate were sufficient to stimulate larviposition, a long held and untested assumption. It is suggested lipids are not required for larviposition but are potent modifiers of the stimulus.

This work is intended to shed light on the biology of an animal that gains precious little respect and normally is ignored or squashed by the vast majority of the population. As I delved into the biology of the animal I could not help but gain an appreciation for it. Adults are marvels of miniaturization, incorporating systems that roboticists only dream of. If observed without stopwatches, computers, and rulers, their behavior does not fit the stereotypical "robotic" image usually associated with insects. If one can put aside their revulsion, even lowly maggots are uniquely fascinating as they gracefully ooze through masses of liver. Disgusting they may be, but their unique adaptations enable them to survive in an incredibly hostile environment with a plethora of competitors, predators, parasites, and noxious chemicals. Despite our best efforts, after more than 80 years, flies have eluded pesticides and swatters alike due to their unique biology. Information in this work is presented not only for illustrating the main points of this thesis but hopefully can be employed as a useful tool for continuing work in the future on what are truly fascinating organisms.

1.5 Biology of *N. bullata*

Neobellieria bullata (Parker) (Diptera: Sarcophagidae) was described more than 90 years ago by Parker (1916) and since then has been used in a wide variety of research. The reasons for its use are fairly simple. It has interesting behavior and physiology, displays representative ontogeny and morphology for flies, is large (10-15 mm long as an adult), comparatively docile and easy to handle, easy to mass rear, and is available from biological supply companies. The biology of this animal is described here to both elucidate and engage the reader. This information was collected from the works of other authors and observations during the course of this research.

Like many species of sarcophagids, *N. bullata* was formerly assigned to the genus of *Sarcophaga* but within the past few years an updated classification has gained recognition. As early as 1916, Aldrich recognized that the genus was not monophyletic. Roback (1954) placed *Sarcophaga bullata* into a new genus *Sapromyia* and Shewell (1987) placed *S. bullata* into the current genus *Neobellieria*. Sarcophagid species are determined using characteristics of the male, which poses problems for field identifications of larvipositing flies. Maggots must be reared to maturity in order to collect the necessary males.

Specimens of *N. bullata* have been collected from across North America north of Mexico. Table 1.1 lists the Canadian provinces and U. S. states that *N. bullata* has been collected from. Some provinces (between BC. and Quebec) and states are missing from this range. It is uncertain whether this is because of gaps in the record or natural habitat preferences of the fly. Virtually no mention is made by any of these authors as to the type of climate this species may prefer, although a few make a passing reference to finding specimens on washed up carcasses near lakes. *N. bullata* is known to overwinter in Illinois (Denlinger, 1972) and probably in other states such as New York as well. The climate conditions of these states, compared to Texas and California, almost certainly are different. Like most aspects of the biology of this animal the environmental conditions the species can tolerate are probably highly variable as well.

First instar larvae are usually deposited on dead animal flesh and the larvae do little else but feed for three instars. Larvae are unsclerotized except for the mouthhooks and cephalopharyngeal skeleton. The white maggots avoid light and attempt to burrow into substrates through pre-existing orifices as much as possible without blocking the spiracles on the posterior end. Larvae are quite sensitive to humidity, preferring low atmospheric humidity and moist substrates. They are capable of tolerating high levels of waste product buildup but extremely high levels are thought to cause maggots to migrate (personal observation; Simms, pers. com.).

Larvae are capable of developing on a wide variety of substrates. Graenicher (1931) believed larval preferences to be much broader than those of adults. *N. bullata* larvae have been recovered from: beetles (Graenicher, 1935); birds (Graenicher, 1931); cockroaches (Graenicher, 1931); crabs (Graenicher, 1931); fresh or decayed pork and beef liver (Kamal, 1958; Knipling, 1936); Friski brand dog biscuits (Kamal, 1958); fish (Graenicher, 1931); marine snails (Graenicher, 1931); snakes (Graenicher, 1935); human excrement (Graenicher, 1935); and can cause myiasis in humans (James, 1947), pigs and cattle (Knipling and Rainwater, 1937). James (1947) suggested that this species was capable of gastrointestinal myiasis but also suggested misidentifications and contamination of specimens had led to misidentification of myiasis producers in the past. In this study, larvae were observed to attempt to keep their posterior spiracles clear even when the maggots had buried themselves deeply into a liver mass. Clearly maggots would have difficulty obtaining enough oxygen to support their high rate of metabolism inside an intestine.

A brief period of wandering occurs after feeding, the length of which depends on ambient light conditions (Denlinger and Zdarek, 1994). After the wandering phase larvae usually burrow into a soft substrate such as the soil surrounding the animal or hide under the carcass. Pupariation will occur even if burrowing is not possible.

Pupariation occurs when the last larval instar contracts longitudinally and expands along its diameter. The larval exoskeleton is then sclerotized to form a dark brown hardened case, the puparium, in which pupation occurs (Chapman, 1969). The pupal stage may last a few days or several months if overwintering. Flesh flies are only capable of surviving winter in the pupal stage (Adedokun and Denlinger, 1984). When they emerge they burst from their puparium by expanding a sac, the ptilinum, from their head using haemolymph.

Pupae are frequently measured for weight. Pupal weights have been recorded from 20 mg (Bennettova and Fraenkel, 1981; Denlinger and Zdarek, 1994; Pappas and Fraenkel, 1977) to

140 mg which Pappas and Fraenkel (1977) regarded as being very large. Adults capable of developing eggs were recorded from these sizes of pupae. In this study pupal weights averaged 150 mg (Section 2) with a maximum weight of 207.3 mg, the largest weight yet recorded for a flesh fly.

Adults emerge in the spring, around May, in Illinois (Denlinger, 1972). Sarcophagids typically have a striped grey and black thorax and abdomen with red compound eyes. Their coloration is set and they are fully capable of running upon emergence. Teneral adults usually expand their wings within 20 minutes and are capable of flight soon after. Adults usually ignore food placed in cages for approximately 24 hours. They begin to mate after approximately 24 hours; females appear to be less receptive after two to three days whereas males appear ready at all ages. Females will mate again 11- 13 days after emergence but it is suspected these females have deposited their first batch of larvae already.

Adults can be kept alive for several days on nothing but sugar and water. Water is the most crucial element for flies because without a source of water adults will die within 24 hours. A protein source is needed for females to develop eggs (Wilkens, 1968; Pappas and Fraenkel, 1977). Females are capable of incipient ontogeny given sufficient larval weight but require a protein source to complete development of eggs (Pappas and Fraenkel, 1977). Even males require a protein source to stay alive over longer periods of time according to De Clerck and De Loof (1983). The number of ovarioles and thus the number of larvae a female will develop depends on its larval weight (Bennettova and Fraenkel, 1981; Kamal, 1958; Pappas and Fraenkel, 1977). The largest pupa recorded by Bennettova and Fraenkel (1981) developed 58 ovarioles per ovary as an adult.

Field populations of *N. bullata* can complete 2-3 generations per year in Illinois (Denlinger, 1972; Denlinger, 1978) and up to four in Ohio (Chen et. al., 1991). Flies reared for this study (Appendix 7.1) were capable of completing a generation, from 1st larval instar to depositing larvae, in 27 to 30 days. In general, larvae usually completed feeding in 5 to 6 days, wandered for about 24 to 48 hours then pupariated 6-8 days after larviposition. The pupal stage lasted a minimum of 11 days, usually 12-14 days. Females were capable of depositing larvae within seven days but the majority would not deposit until 9-10 days after emergence. These observations agree with those of Kamal (1958).

Table 1.1 - Distribution of *Neobellieria bullata*

Province or State	Author
B.C. to Quebec	Stone et. al., 1965
British Columbia	James, 1947
California	James, 1947
Colorado	Aldrich, 1916; James, 1947
Connecticut	James, 1947
District of Columbia	James, 1947
Florida	Aldrich, 1916; James, 1947
Georgia	Aldrich, 1916; James, 1947
Idaho	James, 1947
Illinois	Denlinger, 1972; James, 1947
Indiana	Aldrich, 1916; James, 1947
Iowa	James, 1947
Kansas	James, 1947
Louisiana	James, 1947
Maine	James, 1947
Maryland	James, 1947
Massachusetts	James, 1947
Michigan	Aldrich, 1916
Missisippi	James, 1947
Missouri	James, 1947
New Jersey	Aldrich, 1916; James, 1947
New Mexico	James, 1947
New York	Aldrich, 1916; James, 1947; Stone et.al., 1965
North Carolina	James, 1947
North Dakota	James, 1947
Ohio	Aldrich, 1916; James, 1947
Oregon	James, 1947
Pennsylvania	Aldrich, 1916; James, 1947
Quebec	James, 1947
south to California and Florida	Stone et.al., 1965
South Dakota	James, 1947
Texas	Aldrich, 1916; James, 1947
Utah	James, 1947
Virginia	Aldrich, 1916; James, 1947
Washington	James, 1947

- Locations in North America where *Neobellieria* (=*Sarcophaga*) *bullata* has been collected as stated by the authors

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Chapter 2 Variability in Larviposition Behavior and other Attributes

2.1 Introduction

My research looked at chemical stimuli of larviposition and therefore needed information about larviposition behavior at the level of individual females but such information is almost nonexistent for sarcophagids. Many studies look at ecological and forensic aspects of larviposition although none have focused on *N. bullata* (Catts and Haskell, 1990; Coupland and Baker; 1994; Denno and Cothran, 1975; Denno and Cothran, 1976; Hall and Doisy, 1993; Hanski, 1987a; Hanski, 1987b; Hanski and Kuusela, 1977; Hanski and Kuusela, 1980; Kuusela, 1984; Kuusela and Hanski, 1982; Smith, 1986).

Observations of individual flies have proved useful for the study of myiasis-causing *Lucilia cuprina*, *Phaenicia sericata*, and *Cochliomyia hominivorax* (Barton Browne, 1962; Barton Browne et. al., 1969; Barton Browne et. al., 1990a; Barton Browne et. al., 1990b; Emmens and Murray, 1982; Quattro and Wasti, 1978; Thomas and Mangan, 1989) as well as *Phormia regina*, a carrion feeder (Barton Browne, 1960). Preliminary observations of *N. bullata* were performed by Mitchell and Soucie (1993) whose results indicated the importance of examining individual behavior and variability of characteristics. Their results particularly highlighted the need to collect such information to develop behavioral bioassays which provide unambiguous results.

Carrion feeding flies are by nature highly variable in their biology, especially regarding oviposition or larviposition. Carrion is often scarce, ephemeral, and randomly distributed (Denno and Cothran, 1975; Hanski, 1987b; Kuusela and Hanski, 1982). Flies are subject to intense predation as well as heavy interspecific and intraspecific competition from other invertebrate and vertebrate scavengers (Denno and Cothran, 1975; Denno and Cothran, 1976; Hanski, 1987a; Hanski, 1987b; Hanski and Kuusela, 1977; Kuusela, 1984; Kuusela and Hanski, 1982). As a result carrion feeders need to be flexible in their biology.

Carrion feeding flies do not show the rigid specificities for oviposition or larviposition sites exhibited by plant feeders or parasitoids. Given the chance, flies will oviposit on a wide range of carrion species. *Neobellieria bullata* (Parker) illustrates this particularly well. Larvae have been collected from fresh and decaying animal material of invertebrates (Graenicher, 1931; Graenicher, 1935); vertebrates (Graenicher, 1931; Graenicher, 1935; Kamal, 1958; Knipling, 1936); mammalian excrement (Graenicher, 1931; Graenicher, 1935; Knipling, 1936); and occasionally live animals, including humans (Knipling and Rainwater, 1937; James, 1947). In recent years some disbelief has arisen as to whether any species can be quite this flexible. There are always doubts about the accuracy of earlier identifications, particularly since males are needed to identify species. Sarcophagid larvae including *N. bullata* have been collected from covered, baited containers by Graenicher (1935) and Hallock (1942). This led James (1947) to speculate misidentification of myiasis-causing species can result from sarcophagids depositing into covered jars containing human stool specimens. Hanski (1987b) expressed doubt by stating few flies were likely to breed in both carrion and dung due to nutritional differences. Hanski (1987b) did acknowledge their flexibility by stating some carrion feeding flies may also attack living animals to increase their chances of feeding.

Variability is present in other aspects of *N. bullata* 's biology while other evidence shows some carrion feeding sarcophagids to be specialists. Particularly variable are developmental characteristics. Third instar larvae weighing from 20 to 160 mg successfully emerge as adults and develop viable eggs (Bennettova and Fraenkel, 1981; Denlinger and Zdarek, 1994). Pupae from 8 to 140 mg are capable of developing into adults and producing viable eggs (Bennettova and Fraenkel, 1981; Pappas and Fraenkel, 1977). Females may develop anywhere from 10 ovarioles per ovary to 58 ovarioles per ovary (Bennettova and Fraenkel, 1981) and up to 125 ovarioles total (Pappas and Fraenkel, 1977). Strangely enough other sarcophagid species in California tend to be active over a relatively short duration of the year, from summer to early fall. This may be an example of ecological specialization since many carrion feeding calliphorid species are active over the entire year in the same location (Denno and Cothran, 1975). In a sense some sarcophagids are temporal specialists (Denno and Cothran, 1975), another reason to include variation in the description of their characteristics.

My work in this chapter describes larviposition behavior of *N. bullata* focusing on elements important to investigating chemosensory stimuli. Since variation is an important element of the biology of this animal it was described in detail wherever possible. Data were collected from several sources including experimental trials, dissections, and descriptions obtained while rearing these animals (Appendix 7.1).

Descriptions concentrate on seven major areas. 1) Larviposition behavior of females of different ages. 2) The number of larvae deposited by females during larviposition events. Numbers of eggs or larvae deposited are often described but as Hanski (1987a) pointed out no detailed information exists on how many eggs or larvae are deposited at one time. 3) Larviposition behavior over time. Prior descriptions come from Easton and Feir (1991) who described latency of *Phaenicia sericata* oviposition responses, the time from beginning of a trial to oviposition. Mitchell and Soucie (1993) also described the relationship of taste and larviposition over time. 4) Dissection of adults to determine the progress of oogenesis in females. Bennettova and Fraenkel (1981) and Pappas and Fraenkel (1977) have described egg development in young females but neither examined larval development as flies got older. 5) Data on pupal weights are given, because this is often cited as an example of variability in fly biology (Bennettova and Fraenkel, 1981; Pappas and Fraenkel, 1977). 6) Details of emergence patterns as well as sex ratios of emerging flies since the timing of adult emergences have been described before (Kamal, 1958) and are often used for forensic investigations (Goff and Catts, 1990; Lord, 1990; Smith, 1986). 7) Data on death rates, including sex ratios, of adults are given. Although never specifically stated, flies are assumed to die quickly and in large numbers.

2.2 Materials and Methods

2.2.1 General Information

Observations for all experimental trials were conducted between 10:00 am to 6:00 pm, temperature was between 23.5 °C and 24.5°C and the fluorescent lights of the room were left on for all observations. Females tested were raised according to the methods described in Appendix 7.1. Except for Experiment #1 and where otherwise noted, all females tested were 10-11 days old after emergence from the puparium.

For all experiments, females were observed in chambers illustrated in Appendix 7.2.1, Experiment #6 used a modified version of this chamber (Figure 2.1). This chamber consisted of a clear plastic petri dish and an inverted, transparent plastic drinking glass. Individuals were placed in this chamber along with a single larviposition substrate. Experiment #6 used long strips of plexiglass with holes in it. The glass served as the chamber and isolated one fly per hole. In any trial 13 to 24 females were taken from a group and randomly allocated to a chamber.

Experiments #1 and #2 used pieces cut from pork liver as a larviposition substrate but these were unwieldy and difficult to standardize. In Experiments #3, #4, #5, and #6 a viscous mixture of Sigma liver concentrate (67% w/v), a powder derived from pork liver, proved to be a suitable substitute.

Experiment #1 simply presented a piece of liver in a chamber to females. Experiments #2 to #5 used a plexiglass receptacle (Appendix 7.2.2) coated in red plastic with a 0.5 cm diameter hole bored in the center as a standard delivery system. The hole was filled with about 200µl of substrate. Experiment #6 required different sized holes so long strips of plexiglass were used with different sized holes bored equidistant from each other. This apparatus was not coated in red plastic.

Data was collected from 6 sets of experiments, dissections, and observations taken while rearing flies (Appendix 7.1).

2.2.2 Experiment #1

Females were tested for larviposition response at different ages after emergence from the puparium. Females were tested at seven different ages: 6-7, 7-8, 8-9, 9-10, 10-11, 11-12, and 12-13 days after emergence. These age classes overlap somewhat and are not necessarily mutually exclusive due to the rearing technique used (Appendix 7.1).

Trials were conducted from May/94 to July/94 using pork liver pieces measuring ca. 2x2x0.5 cm in size. Only one liver per trial was used as a source of liver pieces.

Groups of females were watched simultaneously for 66 minutes and were scored for larviposition at two minute intervals. Females were removed from the substrate after completion of the first larviposition event. Larvae were frozen and counted.

Typically females will deposit either directly on or very close to the larviposition substrate (roughly 0.5 cm) while still in contact with at least one leg. Occasionally flies would deposit on the sides of the chamber or a noticeable distance along the base of the chamber from the substrate (>>1.0 cm). A rough approximation of the location of deposition relative to the substrate was also noted and compared between flies of different ages.

2.2.3 Experiment #2

Females were tested for larviposition response using two methods of delivering liver pieces. The first method offered liver pieces measuring ca. 2x2x0.5 cm in size in the center of the chamber (Appendix 7.2.1). The second method offered liver pieces in a plexiglass receptacle (Appendix 7.2.2). One liver per trial was used as the source of liver pieces.

Females from one group per trial were randomly allocated to the two treatments. Females were scored for larviposition at two minute intervals for 66 minutes. Females were removed from the substrate after completion of the first larviposition event. Larvae deposited per fly were frozen and later counted.

2.2.4 Experiment #3

Once standard conditions were determined, it was possible to collate data from three different experiments for which similar controls existed. These data were used as a set of standard measures of response under standard conditions. Larviposition responses were pooled from experiments conducted during three time periods: 1) Aug. 26/94 to Sept. 14/94 2) Nov. 10/94 to Dec 22/94 (pooled from phase 2 in Chapter 3) and 3) May 28/95 to July 22/95.

Standard conditions consisted of: 1) females10-11 days after emergence from the puparium 2) Plexiglass receptacles (Appendix 7.2.2) as a substrate delivery device 3) 67% liver concentrate as a larviposition substrate 4) flies given access to substrate for a period of 66 minutes and 5) flies with no prior larviposition experience ie. the first larviposition event.

Data were collected for the percent of females larvipositing (percent response) and the number of larvae deposited per female for the first larviposition event (#larvae/fly/event).

2.2.5 Experiment #4

Five groups of females observed from Aug. 26/94 to Sept. 14/94 were tested for four consecutive intervals of 66 minutes. Two of the groups observed were also tested in the same manner twenty four hours later.

During the first 66 minute interval, all larviposition events were scored in two minute intervals and the number of larvae deposited per fly was counted. Females were given fresh substrate for each larviposition event. For the three remaining 66 minute time intervals, females were simply scored for a larviposition event. Fresh substrate was given at the beginning of each interval. The same protocol was used for females tested 24 hours later.

2.2.6 Experiment #5

Females observed from Nov. 10/94 to Dec 22/94 (pooled from phase 2 of Relative Roles of Olfaction and Taste) had two behavioral time parameters analyzed: 1) The time from placement in the chamber till the first taste of substrate occurred (time to 1st taste) and 2) The time from placement in the chamber till the first larviposition event occurred (time from 1st taste). Behavioral events were recorded using Observer software (Noldus, 1991).

2.2.7 Experiment #6

Experiment #6 was a followup to Experiment #2 to investigate a mechanism behind the results of that experiment. The data were collected by Blair Faulkner during an undergraduate project.

Different sized holes were drilled into plexiglass sheets measuring 65x10x0.75 cm (Figure 2.1). Five area sizes were used in each sheet measuring 4.9, 19.6, 78.5, 298.7, and 1194.6 mm^2 respectively. The 78.5 mm^2 area corresponds with the area in standard plexiglass delivery devices (Appendix 7.2.2).

Twenty females per trial were randomly allocated to each size class. Females were placed over the substrate under a transparent plastic glass (Figure 2.1). Unlike other experiments, the #larvae/fly/event were not frozen and were counted immediately following the trials. Females were transferred immediately following the first larviposition event. Trials lasted 66 minutes.

2.2.8 Dissections

Females were dissected under a dissecting microscope to determine the contents of the reproductive system. Females were anaesthetized either by chilling in ice first or by freezing overnight. Females were then pinned ventrally under water. The abdomen was cut open between the sternites and pleurites were then gently teased apart to release the paired diverticula of the vagina containing the larvae. Unless dissected dorsally, the diverticula would usually rupture at this point and the larvae would be released. Estimations of the relative proportions of larvae and eggs as well as the number of eggs present were made.

Most females were dissected 10-11 days after emergence but a few females 1-2, 6-7, and 15-16 days post-emergence were also dissected. Most females were fed liver from 3 to at least 5 days after emergence but a few were also fed liver from 4-6 days after emergence. These age classes overlap somewhat and are not necessarily mutually exclusive due to the rearing technique used (Appendix 7.1).

2.2.9 Deposition by Flies in Groups at Different Ages

Groups of flies were fed liver and liver exudate for a protein source to develop eggs (Appendix 7.1). The liver pieces fed to these flies were checked for deposition of larvae and/or eggs 24 hours later. The age classes described overlap somewhat and are not necessarily mutually exclusive due to the rearing technique used (Appendix 7.1).

2.2.10 Pupal Mass, Emergences, and Deaths

Part of the rearing technique required keeping records on pupal mass, emergences, and deaths. These techniques are outlined in Appendix 7.1. Age classes of emerging flies overlap somewhat and are not necessarily mutually exclusive due to the rearing technique used (Appendix 7.1).

2.2.11 Analysis of Data

Description and statistical analysis of data were performed using Microsoft Excel (versions 4.0 and 5.0) and SPSS for Windows (version 6.1). G-tests of independence for a Model II design derived from a formula outlined in Sokal and Rohlf (1981) were programmed on Excel worksheets. Williams' correction (Sokal and Rohlf, 1981) was applied for an adjusted G-statistic which was compared to Excel's built in Chi-square distribution for probability values.

All other descriptions, transformations, and statistical analysis (including Shapiro-Wilks and K-S Lilliefors test for normality, Levine statistic for homogeneity, normal and detrended normal Q-Q plots, t-tests of independent samples, Mann-Whitney U, Kruskal-Wallis one way ANOVA, and parametric ANOVA designs) were performed using SPSS. A two way ANOVA for paired comparisons was used from Sokal and Rohlf (1981) for data collected during Experiment #4 and is discussed in section 2.3.2.4. A nested ANOVA design in the same section was taken from Ott (1993). All statistical analyses will be discussed where appropriate in the Results section.

2.3 Results

2.3.1 Age of Larvipositing Females

Larviposition behavior changes as females get older. Data were taken from observations of females in their rearing cages (Appendix 7.1) and in test chambers (Appendix 7.2.1). Occasionally eggs would be larviposited into liver by females in rearing cages as young as 4-5 days old after emergence. Eggs were the only evidence found of ovi/larviposition at 4-5 days old; females 5-6 days old and older deposited fully functional larvae as well as eggs. Only small batches of eggs and/or larvae (5 to < 300) were found from 4 to 7 day old females. Usually one or two batches were present indicating small numbers of females were responsible. Larger numbers of larvae (100 to >300) were found from females older than 7 days.

Females observed in test chambers began larviposition at older ages than those in rearing cages. Percentage of females larvipositing out of a group from 6-13 days old were observed in Experiment #1 (using test chambers) and the results are summarized in Figure 2.2. In contrast to females in cages, during Experiment #1 flies from 6-9 days old never deposited in test chambers. Larviposition was first observed at 9-10 days after emergence and continued at older ages. Variability among females was large at all ages making it difficult to define an "optimum" age for larviposition. A G-test of independence was used to test the difference in percent response between the age classes of 9-10, 10-11, 11-12, and 12-13 days after emergence. The difference was not considered significant ($p=0.062$). This variability appears to decrease slightly with older flies as seen with 12-13 day old females.

The variability seen in Experiment #1 was compared with that in Experiment # 3 where the larviposition substrate was standardized. Figure 2.3 shows high variability for 33 groups of 10-11 day old females and does not appear drastically different from that seen in Experiment #1. Percent response over 65% was never measured in Experiment #3 although four instances were recorded during Experiment #1. The different substrate used in #3 versus #1 does not seem to reduce variance much but may limit the maximum percent response.

Other evidence indicates behavioral differences among older females. Females from 12-13 days old showed an odd behavior. Occasionally these females would deposit live larvae into the corners of their cages where no obvious larviposition substrates were present. The larvae were deposited in single piles and is likely the work of one or a small number of females. This was never seen with younger females and not all older groups showed this behavior. In one extreme case no examples were observed in a cage of females held until 15-16 days after emergence. Another odd behavior was witnessed with females =/>9 days old of age. After being cooled to < 4^{o}C and warmed up again these flies were seen to larviposit into clean plastic dishes and other apparatus not containing any obvious larviposition substrates

Experiment #1 revealed another difference with increasing age. Usually females deposit maggots directly on or very close (<0.5 cm) to a substrate. Flies of increasing age had an increasing tendency to deposit well out of contact with the substrate (Table 2.1), anywhere from over 1.0 cm away, on the sides, or even on the top of their chambers. No 9-10 day old flies displayed this behavior but older flies did it far more frequently with 20.7% of 12-13 day old flies depositing out of contact.

2.3.2 Number of larvae deposited by females

The number of larva deposited by females was analyzed as the number of larvae (and/or eggs) deposited by each female during a larviposition event ie. #larvae/fly/event. Variance was high although no differences could be detected between individuals, within groups, or between groups. However, in Experiment #2 a distinct difference was detected between two treatments. These flies appear to show some ability to control the number of larvae deposited although the mechanism and reasons could not be determined from the data.

2.3.2.1 age of fly

The age of the fly depositing did not produce any differences (Table 2.2) during Experiment #1. Variation in response was very high, the number of larvae deposited ranged from 2 to 155. Similar ranges were recorded for all age groups. Numbers over 80 were uncommon but did occur repeatedly. Original data was not normally distributed and transformations were not considered satisfactory. Significance was tested using a nonparametric Kruskal-Wallis one way ANOVA for age classes 9-10 through 12-13 days after emergence. At $p=0.5973$ the difference was not considered significant.

2.3.2.2 differences in substrate type

Experiment #2 revealed that given different treatments the #larvae/fly/event can change (Table 2.3). Percent response was the same but different numbers of larvae were deposited per female. Flies deposited a mean of 43.6 larvae on liver chunks versus 10.2 larvae on liver contained in plexiglass receptacles. Original data did not satisfy assumptions of normality or homogeneity of variances between treatment groups and therefore were log transformed. Significance was tested using a t-test for independent samples ($p=0.00001$).

Experiment #6 examined if surface area is one cue used by females to adjust the number of larvae deposited. Figure 2.4 summarizes the results for the five area sizes of substrate. Due to difficulties in rearing large numbers of females for each trial, replicates were difficult to obtain; however some conclusions are possible. Areas of 4.9 mm^2 appeared to be too small to elicit larviposition as only 1 fly deposited. It was also more difficult to collect replicates for 19.6 mm^2 than for larger areas. Differences in #larvae/fly/event were not apparent. A Kruskal-Wallis test of significance ($p=0.6491$) showed no differences in the number of larvae deposited among area classes two through five. Variation appeared to differ slightly between treatment classes (Figure 2.5); however the differences seen here are not nearly as obvious as those seen in Experiment #2.

2.3.2.3 #larvae/fly/event using standard conditions

The standard experimental apparatus eventually used for later bioassays consisted of a chamber (Appendix 7.2.1) and plexiglass block (Appendix 7.2.2). The data on #larvae/fly/event deposited in this arrangement were pooled from Experiment #3 and the results are summarized in Figure 2.5. Females deposit between 1 to 30 larvae 88.8% of the time with the distribution clearly being skewed leftward. However the variability was still high; 11.2% of females deposited > 30 larvae. One female deposited 172 larvae but this number was considered extreme and left out of the graph for the purpose of clarity. The #larvae/fly/event and patterns of variation are very similar to those exhibited by flies using plexiglass blocks in Experiment #2 and flies for all size classes in Experiment #6.

2.3.2.4 between groups, between flies, and within individuals

Experiment #4 recorded multiple larviposition events for females which allowed variation to be analyzed in two different ways: 1) variation between events for different flies and within events for individual females and 2) variation between flies from different groups.

Variation between flies and within individuals was analyzed using a modified two way ANOVA design for paired comparisons outlined in Sokal and Rohlf (1981). Variation between an individual's events (within events) is a problem for analysis since no replication exists by definition (Sokal and Rohlf, 1981). Experiment #4 recorded multiple events for multiple numbers of females; therefore, a two way ANOVA (Table 2.4) was possible for analysis of variation between different individuals. The first four events were analyzed for eight flies. These data were log transformed before analysis which brought the data set within acceptable limits for the assumptions of an ANOVA design. The difference within individual's events was not significant at $p=0.53$. The difference between different individuals had $p=0.086$; however with an adjusted $R^2=0.187$ this model clearly does not account for much variation.

Differences in #larvae/fly/event for females raised in different groups were analysed using a nested ANOVA design (Ott, 1993) (Table 2.5) using data from Experiment #4. Two larviposition events from each fly were used and four flies were nested within four groups. The best transformation for use in the ANOVA design was obtained by taking cuberoots of the original data set. The difference between individuals from different groups was not considered significant at $p=0.28$ with an adjusted $R^2=0.0001$.

2.3.3 Timing of Larviposition

2.3.3.1 larviposition over 2 days

Larviposition is a difficult event to predict with accuracy. Females observed in Experiment #4 remained highly variable over a period of four hours but a few conclusions can be made. As shown in Figure 2.6 the majority of flies that deposited began within an hour of being offered substrate. The response dropped off sharply after the first hour, most depositing within the second hour. Over a period of four hours, approximately 20% more flies larviposited but with a high degree of variability (one hour mean=41.6% SE=9.1; four hour mean=60% SE=8.1). Flies that did not deposit during the first day usually did not larviposit during the second (Figure 2.7).

No reason can be offered for this. Eleven females that did not larviposit on days one and two from Experiment #4 were dissected and all of them contained large numbers of larvae. Results from Experiment #5 (Figure 2.8) show a fairly even distribution of first events over a period of 3960 seconds. A slight bias towards events occurring in less than 1100 seconds is seen.

2.3.3.2 time between taste and larviposition

If larviposition is to occur it usually follows quickly after a taste of the substrate (Mitchell and Soucie, 1993). Results from Experiment #5 (Figure 2.10) confirm this because 61.8% of flies larviposited within 500 seconds of their first taste of substrate. Variability was high with 38.2 % of these flies larvipositing from 500 to 3400 seconds after their first taste. Flies that taste do not necessarily larviposit; 126 of 128 flies tasted substrate yet only 27.3% of females actually larviposited. Although the number of taste events were not systematically recorded, *ad libitum* sampling shows that larviposition could follow few to many taste events. The number of taste events is not likely to be a reliable indicator of larviposition. Flies that tasted many times often did not larviposit.

To put the two measurements in relation to each other, Figure 2.10 and Table 2.6 show time to 1st taste versus time from 1st taste to larviposition for each fly as a ratio of total time available (3960 seconds) in Experiment #5. Variability was high and both events happening anywhere within an hour but taste and larviposition usually occurred close together. Within 396 seconds, 47.1% of flies will taste. Larviposition usually followed soon after with 58.8% depositing within 396 seconds of the first taste.

2.3.3.3 time between 1st and 2nd larviposition events, using 1st and 2nd hour data

From Experiment #4 the time between first and second larviposition events was calculated using those flies that deposited within the first 66 minute interval on day one. Some with a first event during interval one had a second larviposition event in the second interval but no second events were recorded during the third and fourth intervals. For these flies the maximum time possible between their events was calculated.

Results were obtained from 22 females. Most females larviposited twice within the first interval (73%) while the remaining 27% of females had their second event during the second interval. Females in the first interval deposited relatively quickly after their first event (Figure 2.11), with a distribution skewed left. For females with a second event during interval two, it was estimated that they would have had a minimum of 12 and a maximum of 108 minutes for their second event. Unless these females represent a distinct behavioral group, it is likely 108 minutes is a high estimate.

2.3.4 Development of Eggs and Larvae

Due to variability in percent response, several females were dissected to determine the progress of larval development. Table 2.7 summarizes the results of all dissections except for the following notes. Eggs were characteristically long and columnar in shape with a whitish yellow color. They burst easily on contact. Larvae were easy to distinguish as the mouthparts and cephalopharyngeal skeleton were black and the body had visible segments. Larvae were pale white in color. Occasionally a few eggs and larvae were black and this was assumed to be an indicator of disease as no black larvae were ever seen to move. Black eggs and larvae were also seen in batches deposited in trials but were not counted. These were more numerous in older flies from 10- 16 days after emergence.

Larvae and eggs were stored in two sacs in the abdomen. The ovaries are located directly anterior to these sacs but are not actually attached. These sacs are not likely to be the lateral oviducts as these two tubes were empty and join to a common oviduct. The sacs are attached to a tube much farther posterior and it is likely that these sacs are actually diverticula of the vagina. Wilkens (1968) and Bennettova and Fraenkel (1981) stated that larvae are stored in the vagina but neither study noted the presence of sacs.

Under standard conditions (females 10-11 days after emergence, fed from 3 to at least 4 days after emergence) females appeared to be fully gravid and developed large numbers of larvae. In a few rare cases females failed to develop larvae and still had eggs present. Other cases showed some larvae and approximately equal numbers of eggs present. Under these conditions females always developed larvae or eggs. It is not known if development of 1st instar larvae was complete in all females. However many larvae when released from the vaginal diverticula began to crawl while under water.

One to two day old females showed no eggs with yolk or larvae present. Six to seven day old females had eggs complete with a full yolk content. Ten to eleven day old females fed protein one day later showed an almost even mix of larvae to eggs present with only one female failing to develop any eggs. Clearly a high degree of variability is present as some 15 to 16 day old females contained eggs which had not completed embryogenesis .

2.3.5 Pupal Mass

Ovariole development is directly related to the weight of a fly (Pappas and Fraenkel, 1977, Bennettova and Fraenkel, 1981, Kamal, 1958), and since weight is highly variable the number of larvae developed will be variable. The larvae in this study were raised with excess food to develop large flies. Pupae used were on average very large ranging from 126.2 mg to a record 207.3 mg in weight (Table 2.8), sufficient to produce adequate larvae for this study (Pappas and Fraenkel, 1977, Bennettova and Fraenkel, 1981, Kamal, 1958). Although sieving discarded some pupae of sufficient weight (>120 mg) along with much smaller pupae, reduction of overall variance and underweight flies was considered desirable. The rearing method was quite effective since only 3.7% of the 10 414 sieved pupae were discarded.

2.3.6 Emergences

Bioassays required sufficient numbers of females raised under standard conditions (Appendix 7.1). Observations of emerging adults showed sexes did not emerge in equal proportions over time. In order to adapt to this complication, records were kept of the number of emerging females and males as well as pupae discarded before emerging.

Time to emergence of 8273 flies are summarized in Figure 2.12 and Figure 2.13. Figure 2.12 breaks down the sum of adult emergence marked from the first emergence seen in their groups. The majority of adults from groups emerged over a period of 24 to 48 hours. Any pupae remaining were discarded except in a few cases. Some flies emerged after 48 hours but in very small numbers. Some puparia were broken open to determine the reason for this. Some of them were virtually empty and dessicated; others contained a white fluid with few structures formed; others contained white pupae with an identifiable head, thorax, and abdomen; and roughly 50% contained pharate adults far along in the cuticular phase. Some of the puparia may have suffered from damage resulting in dessication. Puparia containing "white" pupae might be in a state of diapause. Denlinger (1972) demonstrated diapause at temperatures around 25^{o}C. Fully formed adults indicate some flies develop slower than others and probably account for some of the incidences of emergences after 48 hours. The discarded puparia represent another example of variability and were present in small enough numbers to allow furthur analysis of the data collected.

Figure 2.13 shows the pattern of adult emergence in terms of time from larviposition. Since some puparia were discarded the extremes of this data are unknown, a bias inherent in the technique. However some trends are evident. Some flies emerge as early as 16 to 17 days after larviposition. The majority of adults emerge from 17 to 21 days with noticeable peaks around 18 to 20 days. Emergence drops off rapidly after this. These results correspond to those found by Kamal (1958).

A total of 4271 females emerged versus 4098 males. Figure 2.14 and 2.15 show the relative proportions of each sex emerging over time. Figure 2.14 shows that females emerge earlier than males on average with 70% of adults emerging within the first day being female. By the second day males emerge in somewhat greater numbers and by the third day males are present in much larger numbers than females. Proportions in terms of age after larviposition (Figure 2.15) show a similar trend with females emerging in greater numbers from 16 to 19 days after larviposition. After this time males appear to emerge in slightly greater numbers and this is particularly evident at 20 to 21 days after larviposition.

Variability of emergences is particularly interesting. As usual variation is high but certain trends can be found. Variation expressed in terms of emergence after larviposition stretches from 16 to approximately 22 days. However groups of flies tend to emerge within a short time period from 24 to 48 hours. Flies reared in the same group appear to be more similar to each other than to flies from different groups.

2.3.7 Deaths

The number of deaths that occured within 10-11 days of emergence were analyzed and are presented in Table 2.9. Flies have a reputation for dying quickly and in large numbers. As a result flies tend to be reared in large numbers to provide sufficient animals for experiments. However, using the techniques in Appendix 7.1, death was not as great a problem as is usually assumed. Several flies that died had trouble with emergence and were often misshapen or very weak. All died within a few days. Other deaths had no discernable reason. Although it is normal to see dead adults in cages it is not common and the numbers were quite low with only 5.7% of adults found dead. The difference between the sexes was not great; 5.1% of females died versus 6.3% of males.

2.4 Discussion

Age of females is an extremely important factor for experimental design. Very young females may larviposit but will often larviposit when embryogenesis is not complete. It is likely that only a small proportion of young females are capable of larviposition and are extreme examples of variation. Larger numbers larviposit at about 9-10 days after emergence; however, factors unrelated to the substrate may affect females as they get older. This may explain larviposition while out of contact with substrates and in cages with no substrates present. Wilkens (1968) showed *N. bullata* females do not resorb eggs. Females older than 9-10 days after emergence show small numbers of black colored larvae, presumably a sign of disease. It is suggested older females may larviposit under less than ideal circumstances in order to dispose of old larvae in favor of developing a new batch. This may explain why some previous authors (Graenicher, 1935; Hallock, 1942) have witnessed some sarcophagid females depositing on covered containers where they could not come in contact with the actual substrate. Females 10-11 days after emergence were selected for experiments. These females deposited while in contact with larviposition substrates most of the time indicating selectivity in comparison to older females. These females also larviposited in sufficient numbers to be useful in later experiments.

Standardizing the larviposition substrate solved several technical challenges. Using Sigma liver powder purchased in one large batch enabled long term experiments using a chemically identical substrate. The standard delivery device solved three other problems. First, it enabled the use of small quantities of substrate (about 200µl) which was essential for testing small quantities of chemically derived fractions. Second, since some unknown factor affects the #larvae/fly/event a standard delivery device acted as a control with untested chemical fractions. Third, variance compared to liver pieces also dropped (SE (liver pieces)=7.4 versus SE=1.4 using liver in plexiglass blocks). Since an adequate larviposition response was maintained using a liver derivative this may provide useful information about the nature of the chemical and physical properties of a larviposition substrate.

The difference between the two treatments in Experiment #2 was completely unexpected. Such a difference has never been demonstrated for a carrion feeding fly before except in population studies using different sized pieces of meat (Easton and Feir, 1991; Kuusela, 1984; Kuusela and Hanski, 1982). In these studies different numbers of eggs or larvae collected were attributed to longer visits by flies on larger pieces of meat (Kuusela, 1984) or greater attractiveness of the substrate (Easton and Feir, 1991). In none of these cases was a difference attributed to variation in the behavior of individual flies except between species. However as Hanski (1987a) pointed out, no detailed information has yet been collected on how many larvae are deposited on one carcass at a time.

Unfortunately the unknown factor affecting #larvae/fly/event could not be resolved using these experiments. Chemical differences do not seem likely for two reasons: 1) the difference was detected using liver pieces cut from one liver and 2) #larvae/fly/event for liver pieces in plexiglass blocks were similar to those obtained with 67% liver concentrate. Simple differences in perceived area seem equally unlikely as our results did not show any changes in larviposition using increasing area sizes of substrate. Flies will not walk through liquids, such as 67% liver concentrate, for simple mechanical detection of size differences which leaves visual cues as a method. Visual detection of size differences therefore does not seem likely to be a major factor. Flies were often observed to circle a substrate while tasting. In the case of liver pieces flies also walk over the substrate. One explanation is that flies detect some difference between a large liver piece and those in the plexiglass blocks by assessment of size while walking. Another alternative is that they detect a difference between substrates using tactile information. Tactile cues modifying the perception of flavor are well known in vertebrates. Perception of fats involves tactile differences (Ramirez, 1994). Astringency also modifies the perception of taste by causing tactile differences in substrates (Breslin et. al., 1993). Tactile differences modify oviposition responses of the myiasis-causing screwworm, *Cochliomyia hominivorax* (Hammack, 1991) but the mechanism is not known. Flies may also evaluate a substrate using olfaction and taste as well as tactile cues from various locations on the body, particularly the legs.

Time is one of the most important factors in experimental design. A period of about one hour was judged sufficient for the purpose of evaluating larviposition responses; in particular when frequency of larviposition in response to different conditions is a relevant variable. However, determining maximum larviposition response on substrates is likely to be far more difficult. For most practical purposes longer periods of time do not result in larger numbers of females larvipositing. Some gravid females containing larvae would not larviposit even when tested on two occasions over 48 hours. Perhaps once larvae are developed, the availability of substrate is not the only factor regulating larviposition.

Latency is another aspect of time relevant to larviposition that must be considered in experimental design. Our results agree with those of Mitchell and Soucie (1993) who showed flesh flies exhibit short periods of latency especially with respect to taste, yet the high variance remains a major concern. If latency is a relevant variable, then a sufficient amount of time must be allowed. Easton and Feir (1991) found this to be a pertinent variable where different types of larviposition substrate were concerned and they allowed from 60 to 90 minutes for oviposition. In Experiment #4 latency between 1st and 2nd events was examined. The duration of this interval could not be accurately determined in some cases since some 2nd events could not be recorded within the first hour. If latency between 1st and 2nd events is considered relevant more time should be alloted to answer the question.

No specific work has been performed to investigate the relevance of duration and frequency of taste events to larviposition. Mitchell and Soucie (1993) systematically recorded taste events but did not correlate them with larviposition events. In this study, frequencies of taste events were not systematically recorded but were sampled *ad libitum*. From this, I think that frequency and duration of taste events are not an important indicator of larviposition.

Pupal weights highlight the extreme variability that exists in the biology of this animal. Previous authors have recorded weights from eight to what was regarded as an extremely large140 mg (Bennettova and Fraenkel, 1981; Pappas and Fraenkel, 1977). Masses recorded in these trials show these previous records to be low compared to what can be achieved. Their insects were raised in comparatively high densities and may have been resource limited. Nevertheless, these lower pupal masses might be closer to what is achieved in a highly competitive natural setting. Large sized pupae are desirable in an experimental setting where #larvae/fly/event are considered a relevant variable. In these experiments, flies had more than sufficient numbers of larvae to deposit in multiple events of larviposition. With the variance shown by these animals, females from previous work would not likely have had sufficient numbers of larvae to show the extremes between different conditions or even under standard conditions. Screening of pupae also helps to reduce overall variance of mass in a population.

Tracking emergences proved valuable for two reasons. First, knowing the sex ratios was useful when obtaining sufficient numbers of females for experimental designs was necessary. Clearly females tend to emerge earlier than males. Second, tracking the emergences also demonstrated differences between groups in terms of emergences. Whether looking at total emergence from groups or sex ratios, the variation seen within groups was less than that between groups. This was a difference not seen using other types of information. Although percent response varies greatly between groups, these differences could not be attributed to similarities between individuals as opposed to chance. ANOVA designs to analyze differences between #larvae/fly/event from members of different groups also failed to reveal any significant differences. Similarities of individuals raised in the same group is known from previous work. An unusual example of this was shown by van der Molen et. al. (1985) by analyzing differences of neurophysiological responses in blow fly leg hairs. He mentioned in passing that individuals from the same cage have responses more similar than those from different cages. It is still unknown whether these differences between groups are a result of small changes in laboratory climate or genetic similarities in collected larvae .

Nothing has been published about the death rates of flesh flies. It is generally accepted that flies die rapidly and in large numbers. Given their short life spans of about 60 days (assuming no diapause) this may seem obvious. In the course of this study, however, flies died continuously but in small proportions. Flies appear capable of remaining healthy when reared in clean conditions and low densities.

From a broader point of view, one of the most interesting aspects of *N. bullata* 's biology is the variability. In retrospect, this should not be too surprising as high variability under natural conditions is obvious. This variability would be easy to attribute to various factors such as climate and variance between populations. However, in this investigation great care was taken to study this animal in a controlled environment both with regards to rearing and experimentation. The experimental cultures were taken from an inbred colony maintained for several years under homogeneous conditions. The presence of such variation only serves to highlight its importance in the biology of the animal and might even suggest developmental mechanisms to maintain it. Carrion feeding flies live in a hostile and unpredicatable environment where high variability in several life history traits is clearly advantageous.

Table 2.1 - Percentage of flies which larviposited greater than 1.0 cm away from liver chunks.

age (days after emergence)	n	larviposition off substrate	%
9 - 10	12	0	0.0
10 - 11	46	2	4.3
11 - 12	25	2	8.0
12 - 13	29	6	20.7

Table 2.2 - Number of larvae deposited by females at different ages [a] during their first larviposition event (using liver chunks as a substrate).

Age (days after emergence)	n	mean	SEM	min	max
9 - 10	12	54.3	14.1	6	155
10 - 11	44	43.6	4.6	6	141
11 - 12	28	55.8	7.2	2	132
12 - 13	33	50.7	6.6	5	150

[a] n.s. (Kruskal-Wallis, $p=0.5973$, $\chi^2=1.8817$, d.f.=3)

Table 2.3 - Summary of larviposition responses for flies using liver chunks and liver pieces in plexiglass receptacles.

Larviposition substrate	Percent response [a]		#larvae deposited per female in first event [b]			
	n	# (%) responding	mean	min	max	SEM
liver chunks	44	19 (43.2%)	43.6	7	141	7.4
plexiglass receptacles	46	19 (41.3%)	10.2	3	28	1.4

[a] n.s. (G-test of independence, p=0.9092, Gadj=0.8582, d.f.=1).

[b] difference significant (t-test of independent samples using log transformed data set, p=0.0001, t=6.53, d.f.=36)

Table 2.4 - Analysis of variance for the number of larvae deposited between individual female's events and between events of different females. The number of larvae from the first four events of eight females were used. Data were log transformed for statistical comparison.

Source	d. f.	M. S.	Prob>F
Model	10	0.27	0.14
event (within flies)	3	0.12	0.53
fly # (between flies)	7	0.33	0.09
error	21	0.16	
Total	31	0.19	

$R^2 = 0.449$
$R^2_{adj} = 0.187$

Table 2.5 - Analysis of variance for the number of larvae deposited during larviposition events between females raised in different groups. The number of larvae deposited in the first two events of four females per group of four groups were used. The cuberoots of data were taken for statisical comparison.

Source	d.f.	M. S.	Prob>F
Model	15	0.31	0.73
- group	3	0.6	0.28
- fly # nested within group	12	0.24	0.85
error	16	0.43	
Total	31	0.37	

$R^2 = 0.404$
$R^2 adj = 0.0001$

Table 2.6 - Relationship between tasting and larviposition by time (s). Time to 1st taste refers to amount of time taken by females to taste from start of experiment. Time from 1st taste to larviposition refers to amount of time taken by females to initiate their first larviposition event after their first taste of substrate. Trials were 3960 s in length. Time taken for variables were categorized into intervals of 0 to 396 s (10% of the interval) and 396 to 3960 s (90% of the interval). Females had a tendency to taste within 396 s . Larviposition has a tendency to follow soon after the first taste. Many females responses took considerably longer and were spread over 396 to 3960 s.

Response	Time (s)	
Variable	0 to 396	396 to 3960
time to 1st taste	47.1%	52.9%
time from 1st taste to larviposition	58.8%	38.9%

Table 2.7 - Contents of vaginal diverticula of females. Females of different ages were dissected and contents of their vaginal diverticula were released. Most females were given liver to feed on from 3 days to at least 4 and no longer than 6 days after emergence. Females were not included in results if any evidence existed of prior larviposition. One group of females dissected at 10-11 days were not fed liver until 4 days after emergence. Estimations of the amount of larvae and eggs contained were made.

Age of dissection (days after emergence)	Ages fed protein (days after emergence)	all or mostly larvae	all or mostly eggs	50:50 larvae: eggs	no larvae or eggs	Total
1 to 2	n/a	0	0	0	6	6
6 to 7	3 to 4	0	4	0	0	4
10 to 11	4 to 5	4	4	0	1	9
Standard: 10 to 11	3 to 4	36	2	4	0	42
15 to 16	3 to 4	6	0	3	1	10

Table 2.8 - Pupal mass (mg): pupae retained for trials versus discarded. Pupae were sorted for size using a plastic sieving device called the Pupa Scooper (Appendix 7.1). Pupae retained in the Pupa Scooper were considered to be of adequate size. Pupae not retained in the Pupa Scooper were discarded.

	Retained	Discarded
n	75	43
mean	157.2	125.2
median	154.8	126.8
standard deviation	19.2	18.1
SE	2.2	2.8
minimum	126.2	61.1
maximum	207.3	153.0

Table 2.9 - Deaths of adults in 10-11 days after emergence. Adult cages were checked daily for dead flies from Apr 18/94 to Sept 22/95. Deaths were only included for a period of 10-11 days after emergence of groups.

	Emergences	**Deaths**	**%**
Total	5893	333	**5.7**
Female	3058	156	**5.1**
Male	2830	177	**6.3**

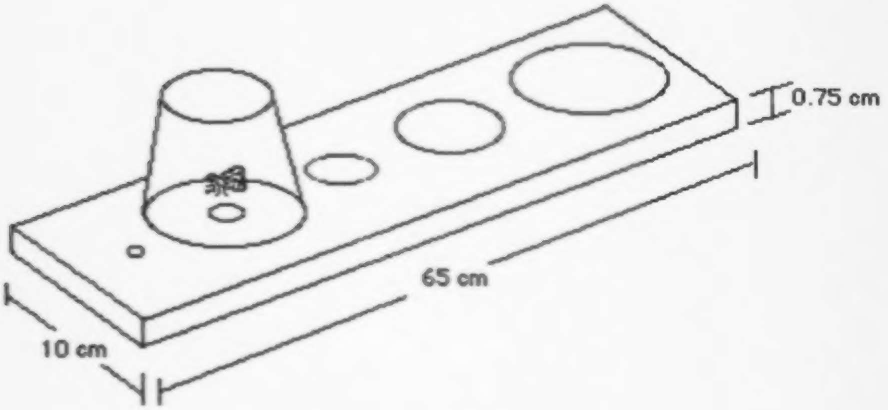

Figure 2.1 - Experiment #6 Apparatus

- plexiglass sheet composed of two layers: top layer bored out for wells, bottom layer glued to top using cyanoacrylate

- plexiglass is transparent and not coated

- 20 flies observed on 4 plexiglass sheets

- flies contained in transparent, plastic cups inverted over different treatment sizes

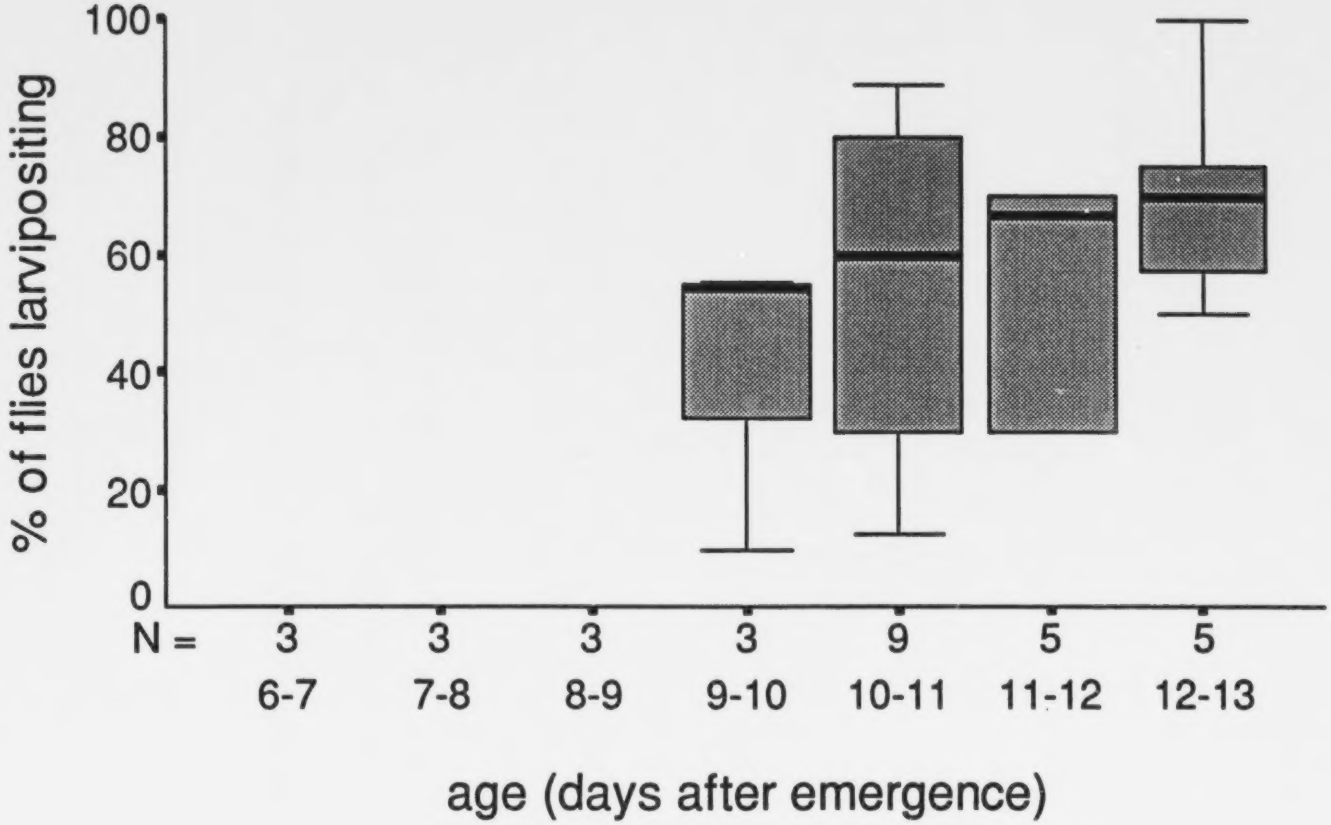

Figure 2.2 - Percent of flies larvipositing by age. Data were taken from the percentage of flies larvipositing out of groups of flies that were reared together. The difference between age classes (9-10 through 12-13 days after emergence) responding was not significant (G-test of independence, p=0.062, Gadj=7.335, d.f.=3). Vertical bars represent min/max values that are not outliers; horizontal lines of boxplots represent the median.

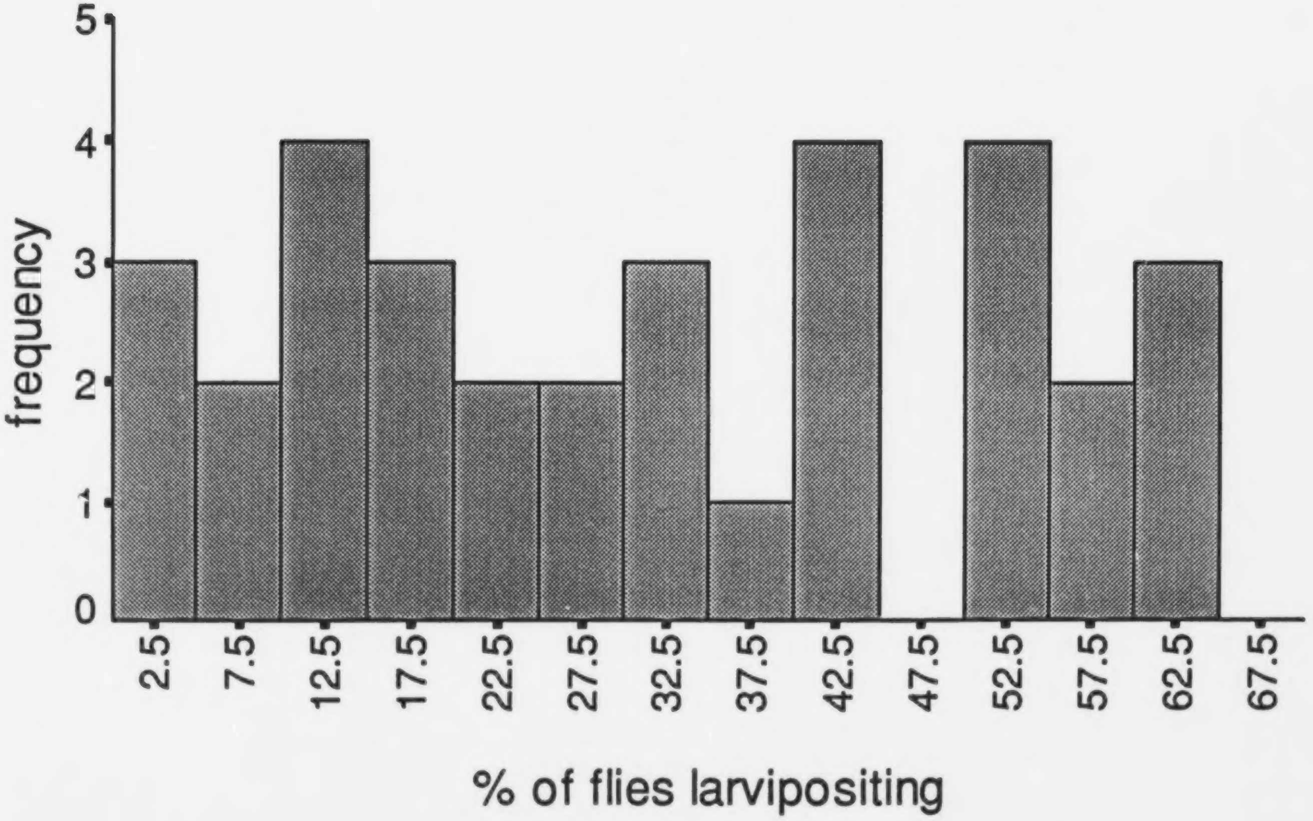

Figure 2.3 - Percent of flies larvipositing under standard conditions. Standard conditions consisted of 67% liver concentrate in a plexiglass receptacle as a substrate, females were 10-11 days after emergence with no prior larviposition experience, and trial duration was 66 minutes. Data were taken from the percentage of flies larvipositing out of groups of flies that were reared together (mean=30.1, SD=19.85, n=33).

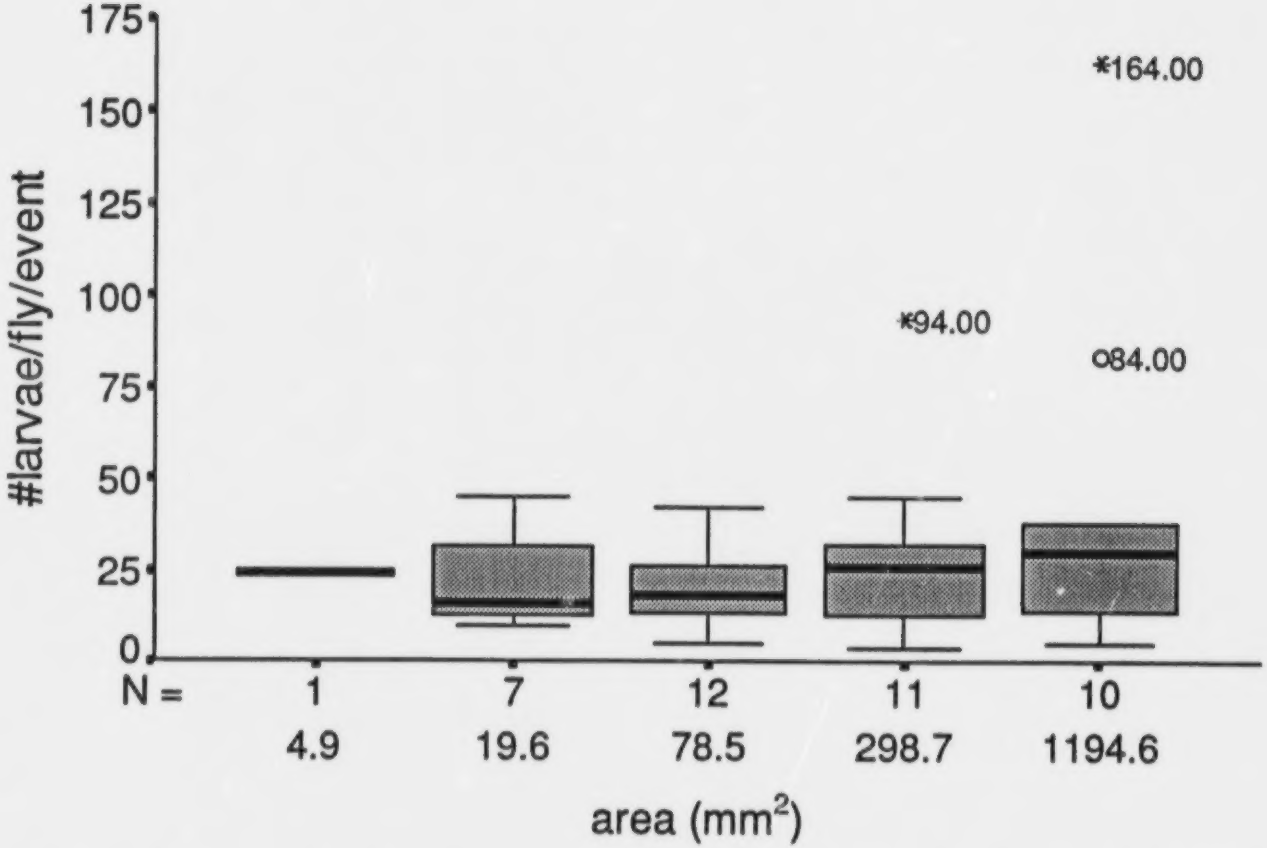

Figure 2.4 - The number of larvae and eggs deposited by each female in her first larviposition event using different surface areas of substrate. Substrates consisted of 67% liver concentrate contained in different area sizes of hole in a plexiglass sheet. Vertical bars represent min/max values that are not outliers; horizontal lines of boxplots represent the median, o=outlier, *=extreme values.

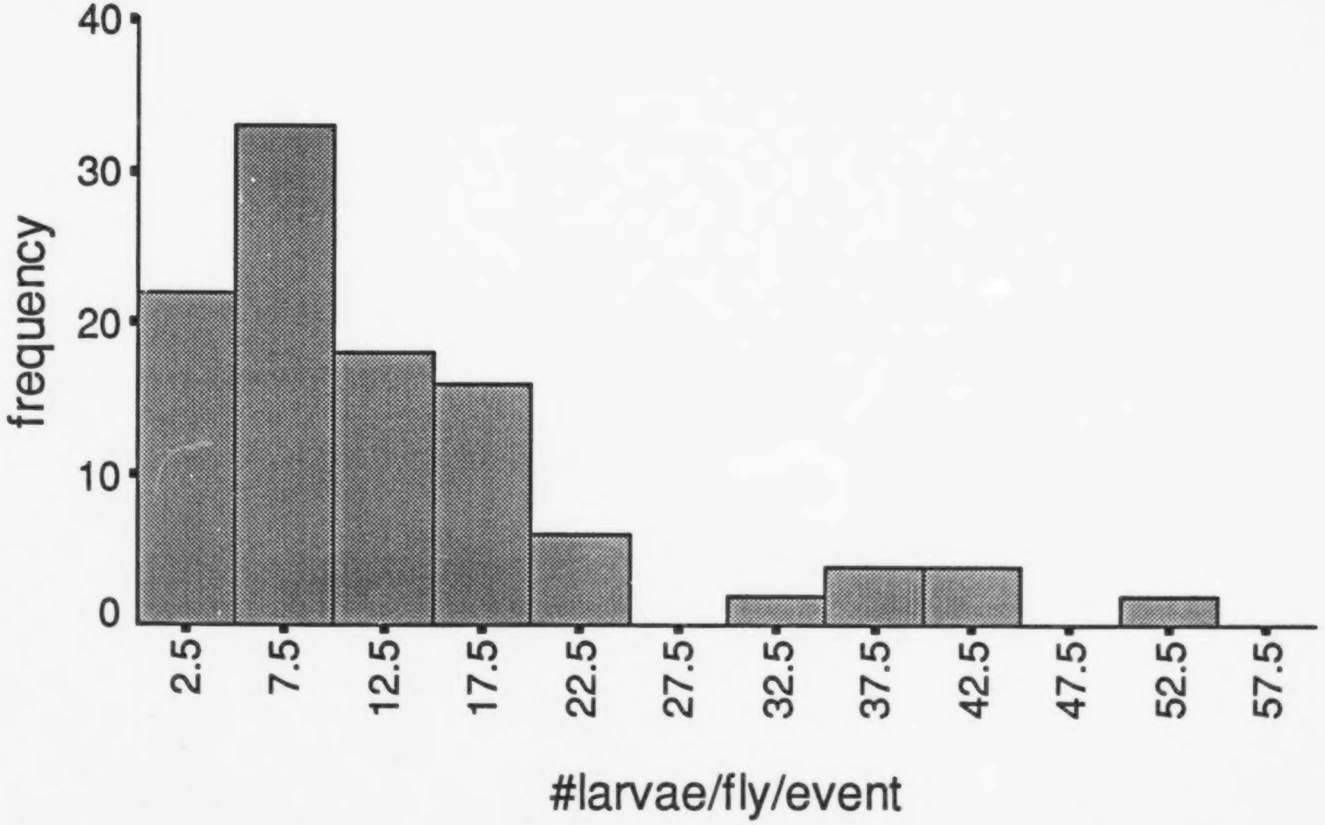

Figure 2.5 - Number of larvae and eggs deposited per female during her first event of larviposition under standard conditions. Standard conditions consist of 67% liver concentrate in a plexiglass receptacle as a substrate, females were 10-11 days after emergence with no prior larviposition experience, and a trial duration of 66 minutes. (mean=12.8, SD=11.69, n=107)

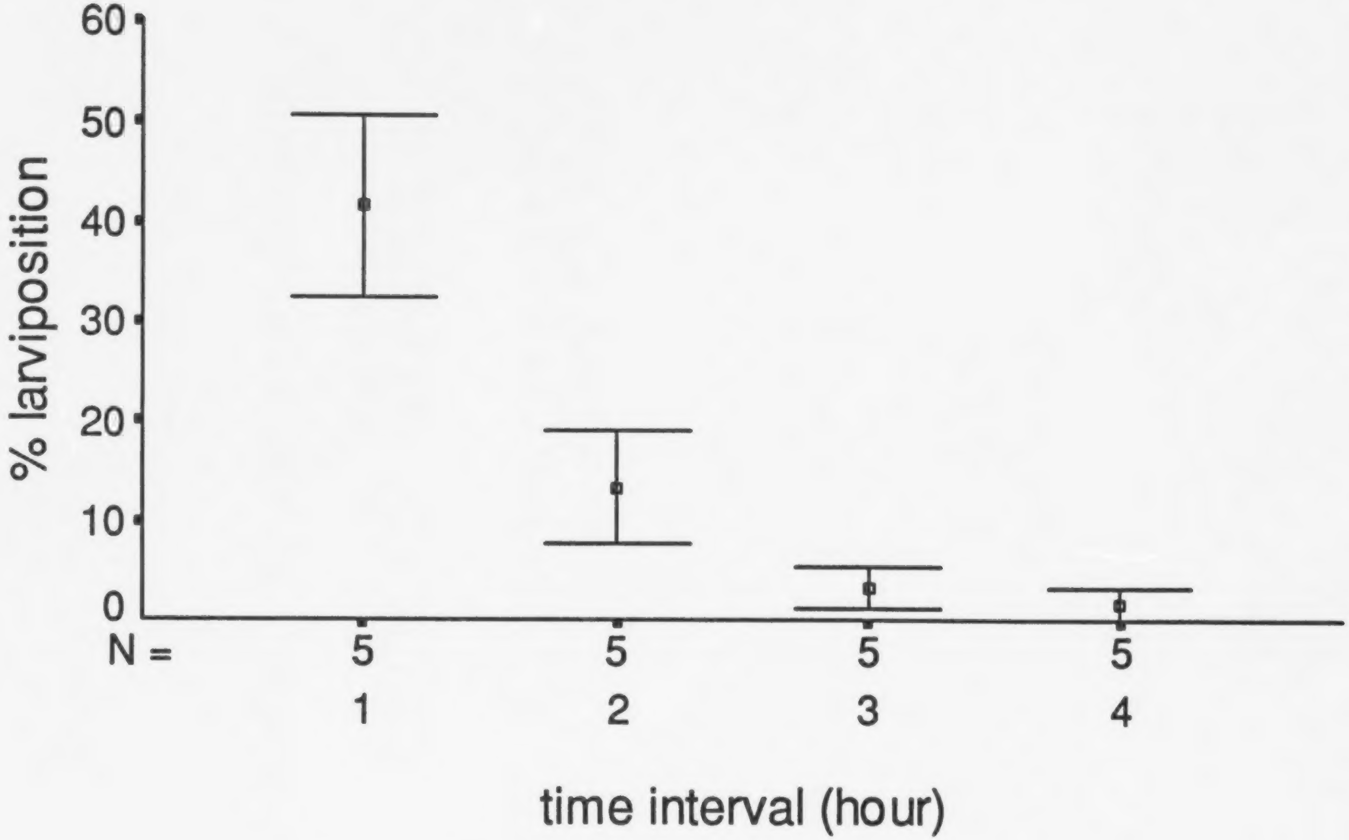

Figure 2.6 - Percentage of flies depositing for the first time during the first day over four one hour intervals. Data were taken from the percentage of flies larvipositing out of groups of flies that were reared together. Error bars are one SEM.

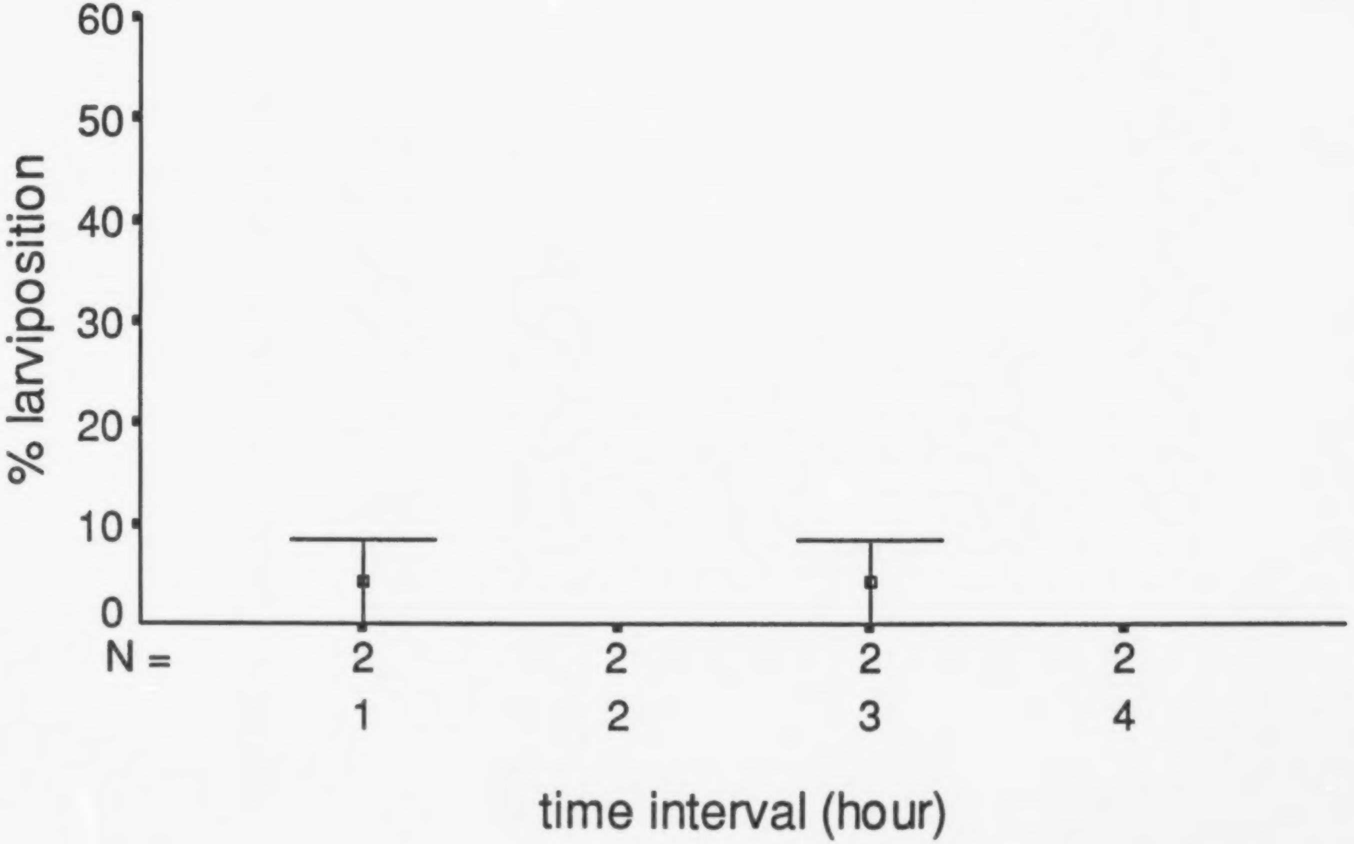

Figure 2.7 - Percentage of flies depositing for the first time during the second day over four one hour intervals. Data were taken from the percentage of flies larvipositing out of groups of flies that were reared together. Error bars represent one SEM.

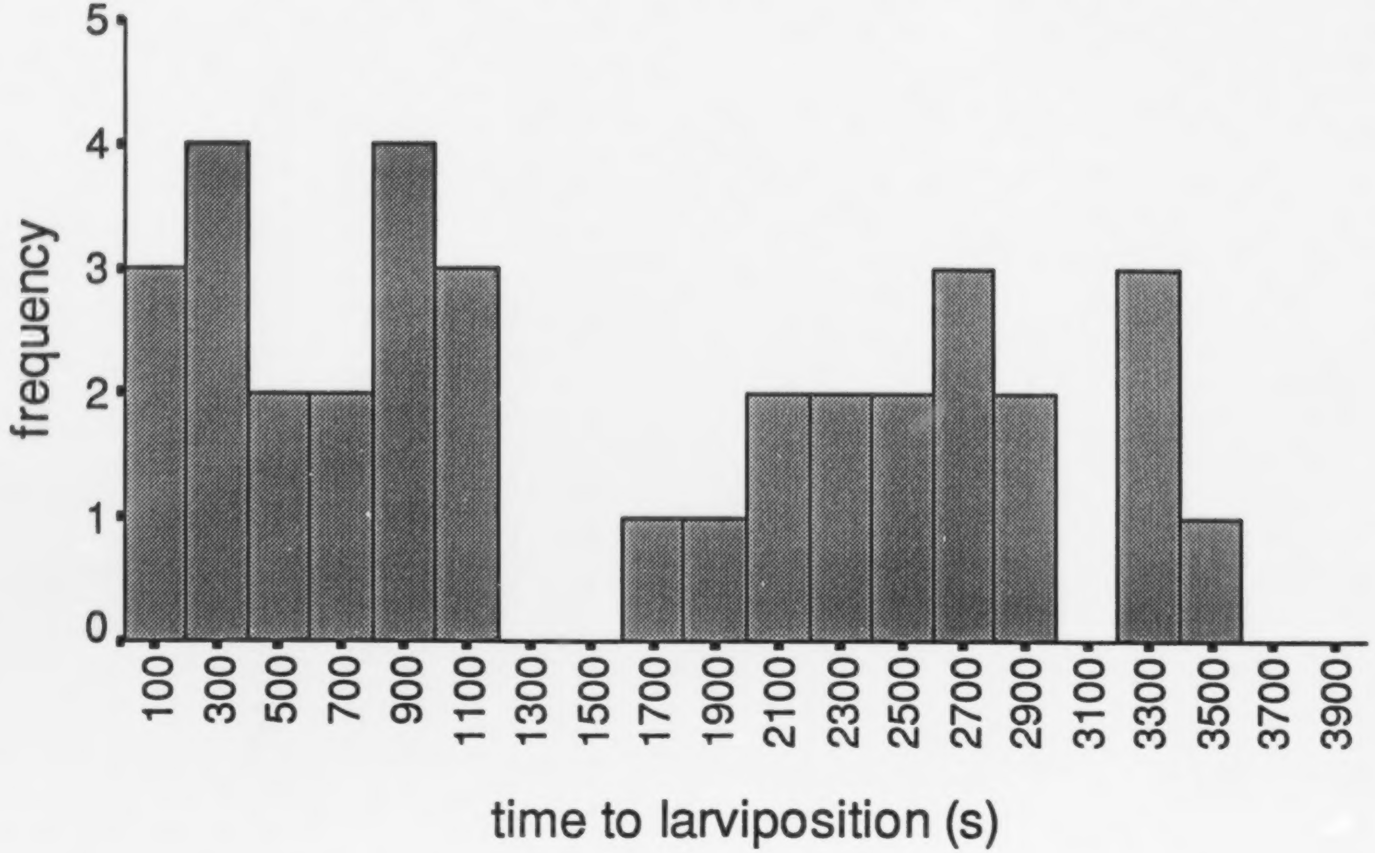

Figure 2.8 - Time to the first larviposition event within one hour. Events on histogram refer to the time taken by females to larviposit from the start of the trial. Trials lasted 3960 s (mean=1586.0, SD=1122.1, n=35).

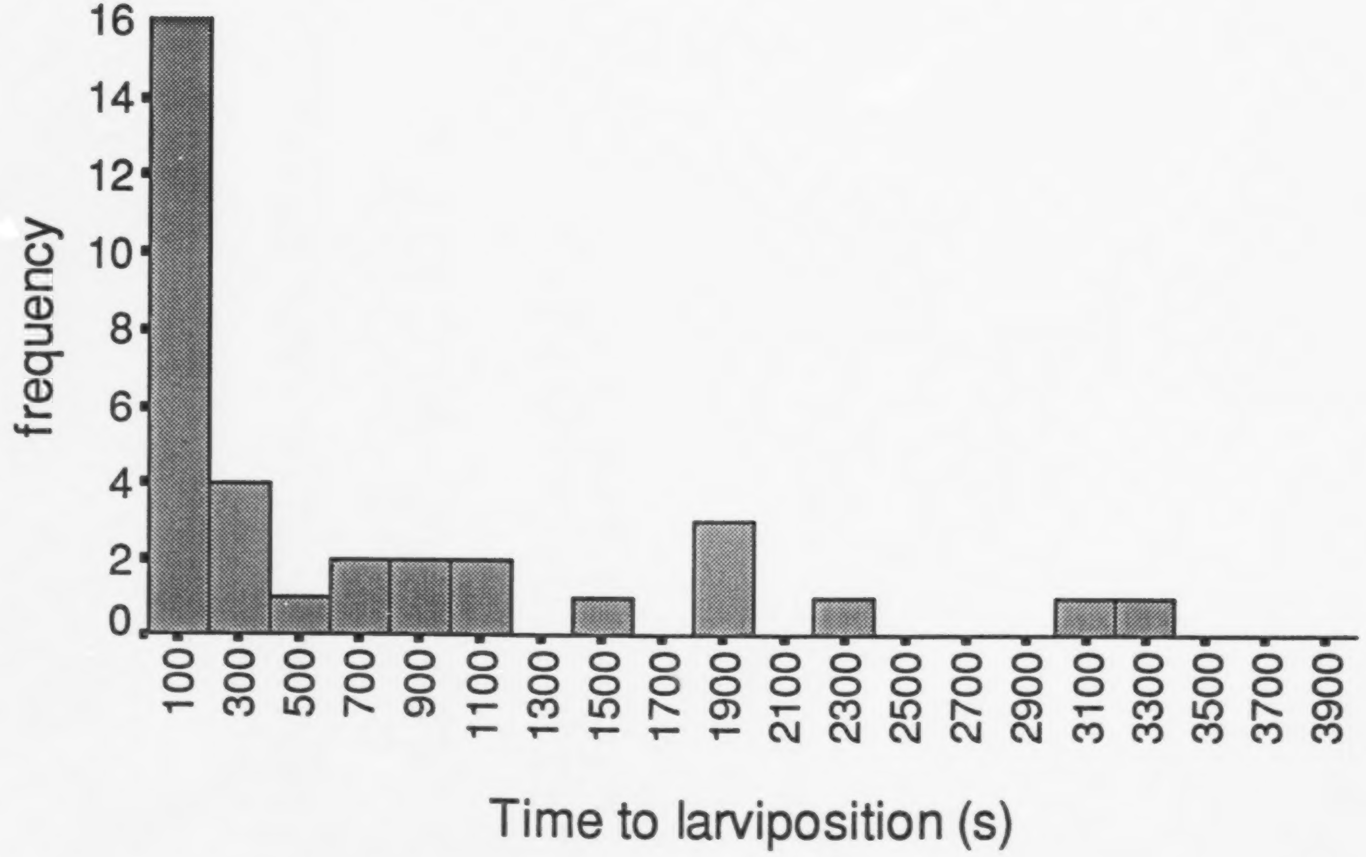

Figure 2.9 - Time to the first larviposition event after the first taste of 67% liver concentrate. Trials lasted 3960 s (mean=699.0, SD=933.13, n=34).

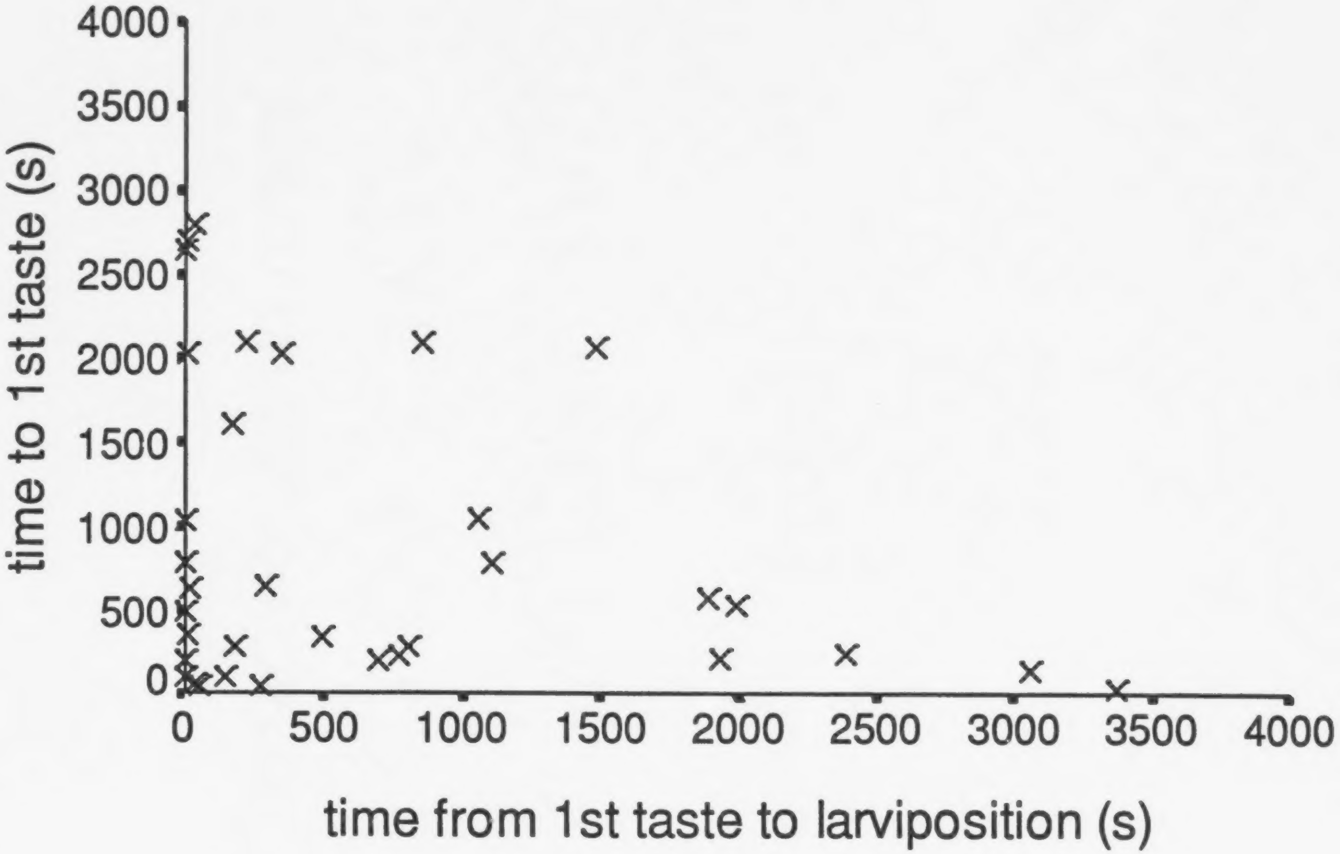

Figure 2.10 - Time to the first taste of 67% liver concentrate versus the time from the first taste to the first larviposition event. Trials lasted 3960s; x=time events of a fly (n=34).

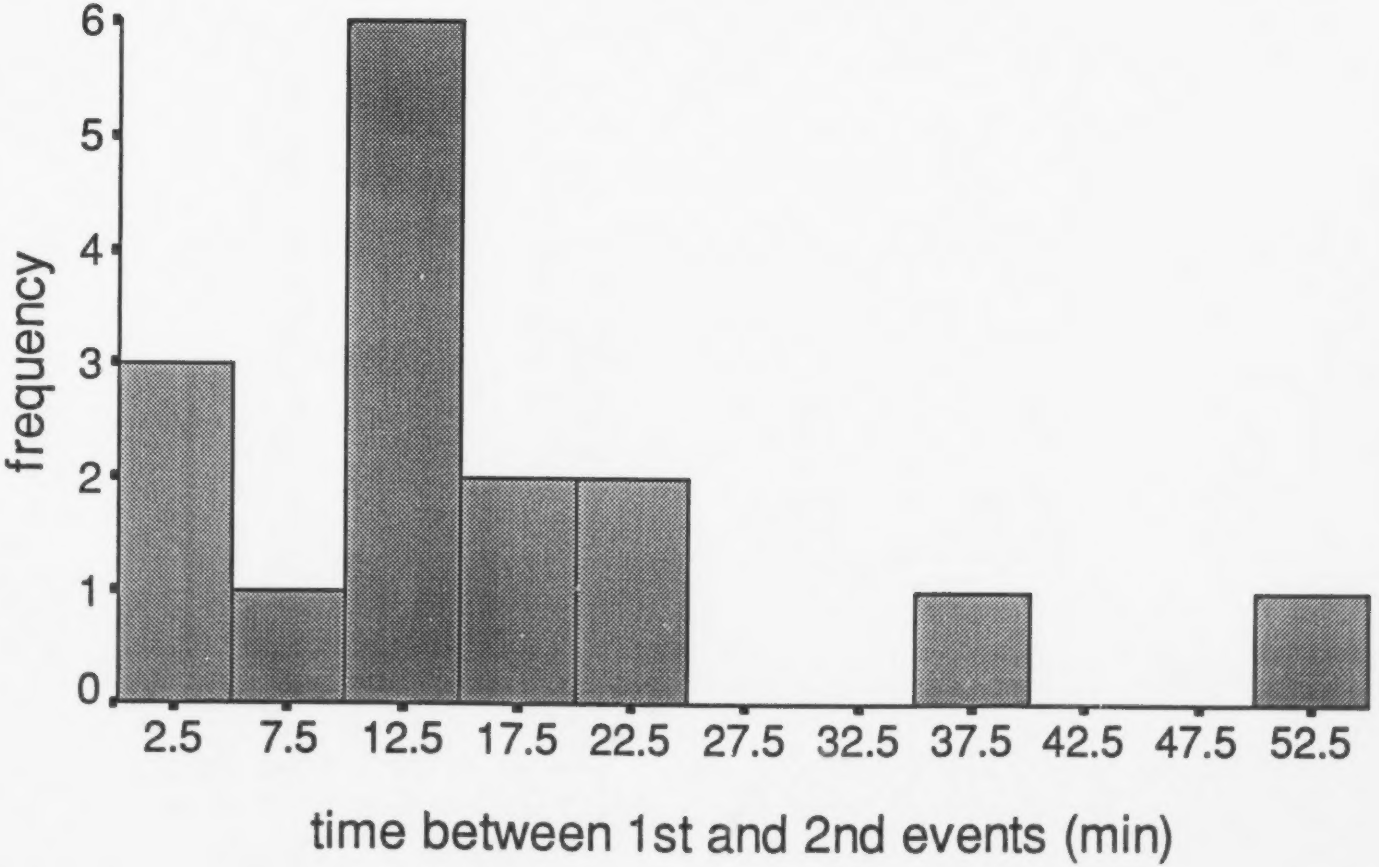

Figure 2.11 - Time between the first and second larviposition events during the first one hour interval. Data were included only if females had their second event during the first one hour interval on the first day (16 out of the 22 flies that larviposited twice) (mean=15.5, SD=12.45, n=16).

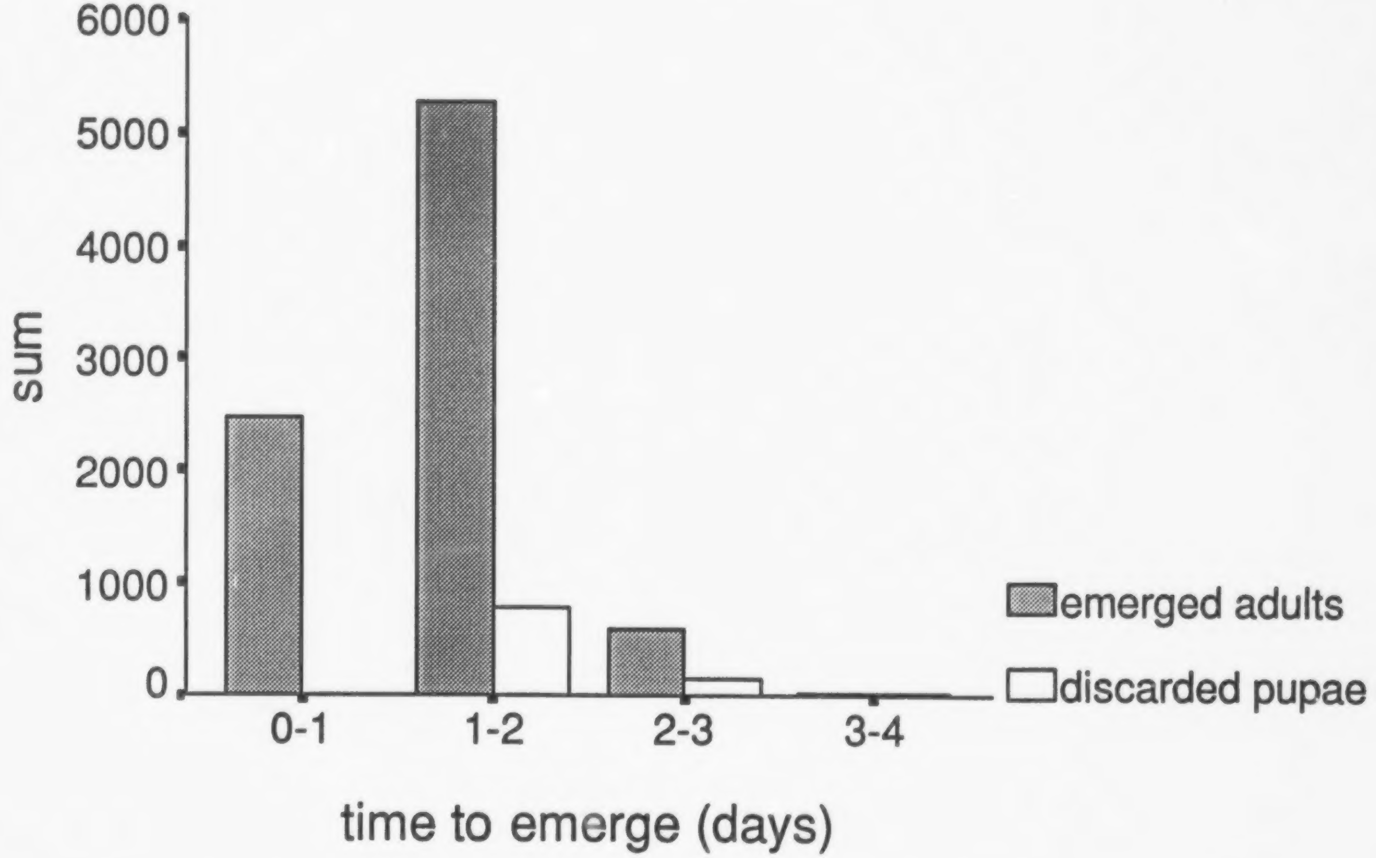

Figure 2.12 - Sum of adults emerging over time measured from the first occurrence of emergence seen in a group of flies reared together. Adults were counted from April 18/94 to Sept. 22/95. The majority of adults emerged within 48 hours and small numbers of pupae were discarded afterwards (n=8273).

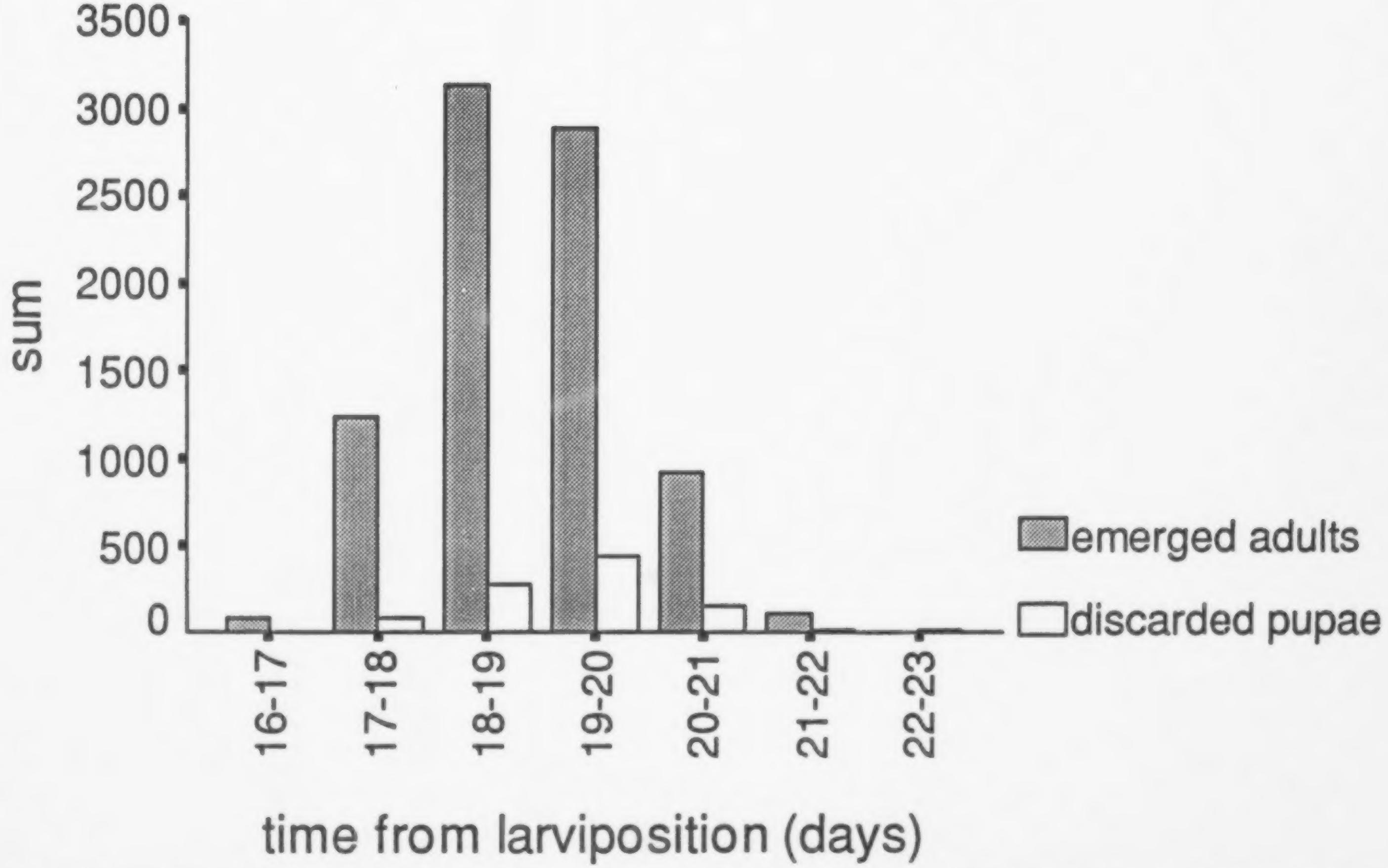

Figure 2.13 - Sum of adults emerging over time measured from the larviposition date of a group of flies reared together. Adults were counted from April 18/94 to Sept. 22/95. The majority of adults emerged from 17 to 21 days after larviposition. Small numbers of pupae left over were discarded (n=8273).

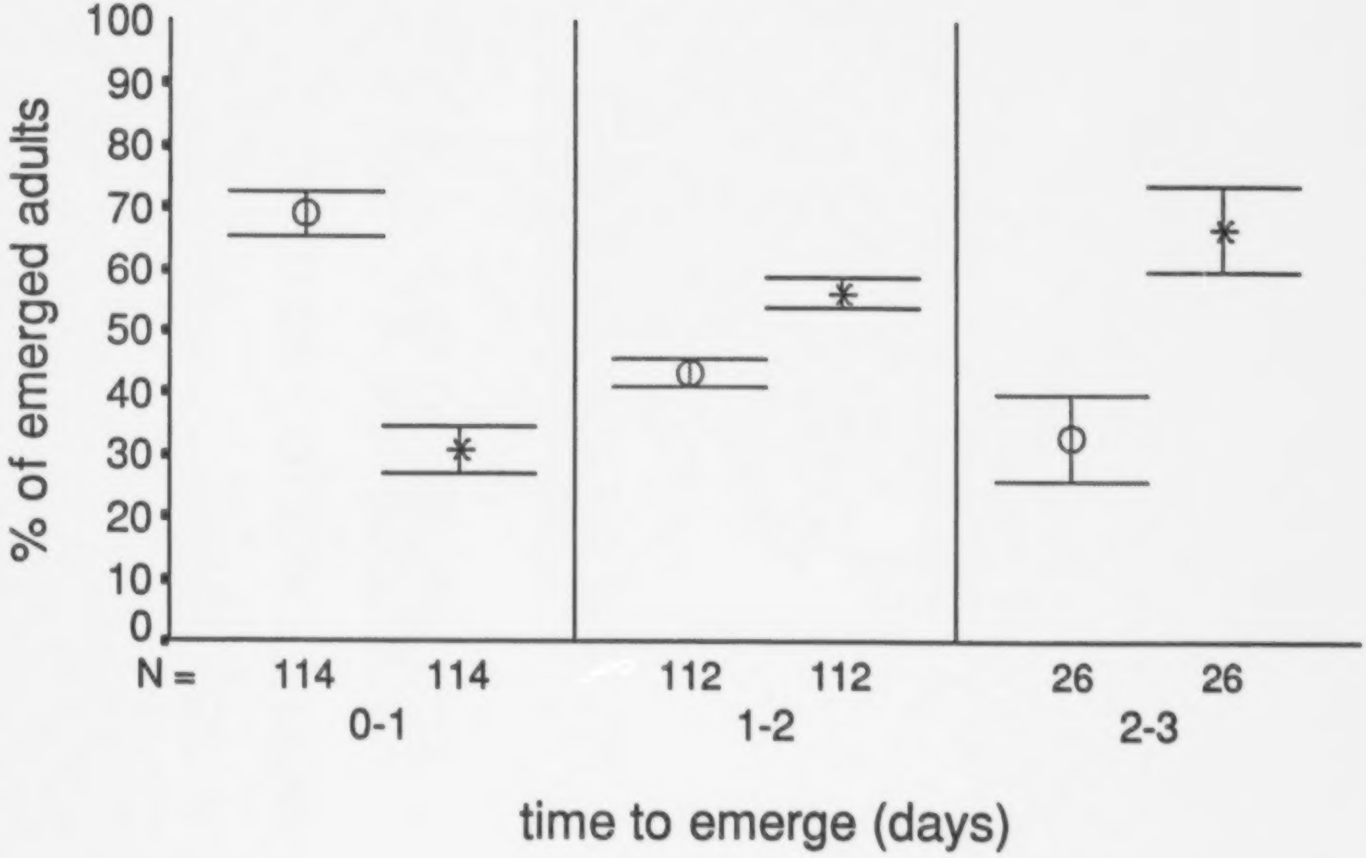

Figure 2.14 - Sex ratio of adults emerging over time measured from the first occurrence of emergence seen in a group of flies reared together. Adults were counted from April 18/94 to Sept. 22/95. Error bars show 2 SEM, o = female emergence, * = male emergence (n=8273).

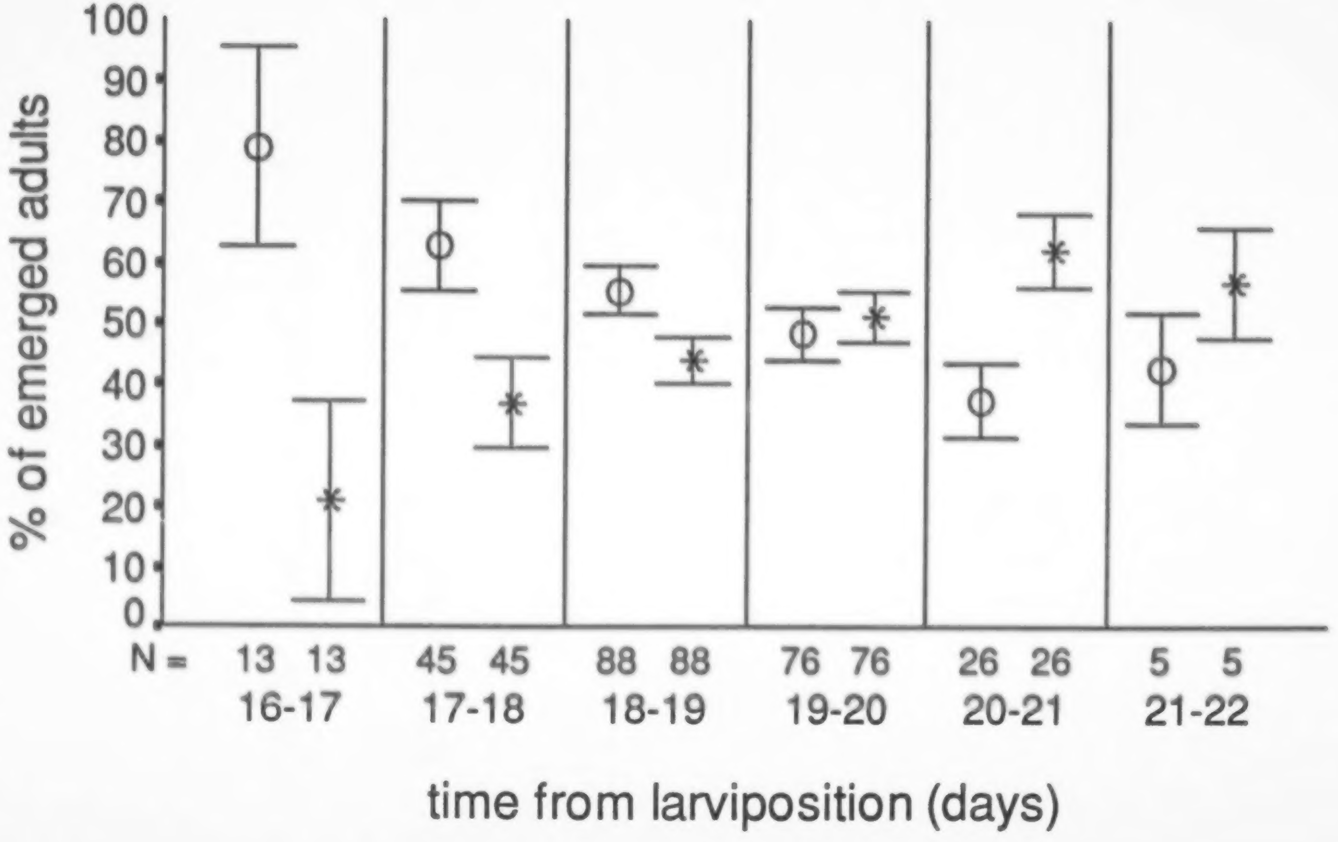

Figure 2.15 - Sex ratio of adults emerging over time measured from the larviposition date of a group of flies reared together. Adults were counted from April 18/94 to Sept. 22/95. Error bars show 2 SEM, o = female emergence, * = male emergence (n=8273).

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Chapter 3 Relative Roles of Olfaction and Taste

3.1 Introduction

The importance of olfaction to flies is well documented, especially for attraction. For a carrion feeding fly the aroma of a rotting corpse is a powerful attractant. Lord (1990) cites the case of a murder victim found in a well covered with debris. Flies were hovering over the location in response to the odors emanating through the debris. However Cragg (1950) showed attraction of flies and inducement of oviposition are not necessarily mediated by the same chemical stimuli, indicating two distinct phases of oviposition or larviposition behavior. Inducement is a complicated aspect of oviposition that involves both of the chemical senses, taste and olfaction, as well tactile cues in some cases.

The relative influence of taste and olfaction varies greatly with different species of flies. *Phormia regina* can use olfactory information almost exclusively and will oviposit in response to the odor of an oviposition medium (Barton Browne, 1960). Olfactory stimuli are also the primary chemical cues for *Lucilia cuprina* but in this case tarsal contact with water is required (Barton Browne, 1962), indicating a need for contact chemoreception. In addition, olfaction is important to the primary screwworm, *Cochliomyia hominivorax*, but contact chemoreception plays an important role in modifying the response as does tactile stimulation (Hammack, 1991).

Literature regarding stimulation of *Neobellieria bullata* is somewhat conflicting. Mitchell and Soucie (1993) reported that this fly will not larviposit without tasting a suitable substrate first using the labellum, implying a major role for taste input. However the authors also indicated a possible role for olfactory input in mediating a central excitatory state (CES). Preliminary results suggested that this CES was induced when flies were placed in the presence of odors without actually contacting the substrate. Olfactory stimuli were thought to "precondition" females to the presence of a substrate.

Earlier work on members of the Sarcophagidae placed heavy emphasis on olfactory stimulation of larviposition. As early as 1921, Wardle believed sarcophagids oviposited in response to olfactory cues whereas calliphorids deposited in response to gustatory ones. Graenicher (1935) observed *Sarcophaga bullata* dropping larvae through cheesecloth covered bottles in response to the odors of decaying beetles. Sarcophagid larvae including *N. bullata* were recovered from covered, baited containers by Graenicher (1935) and Hallock (1942). James (1947) speculated that misidentification of myiasis-causing species can result from sarcophagids depositing into covered jars containing human stool specimens. Denno and Cothran (1976) believed that contact with a substrate was not necessary since flies would deposit their larvae by dropping them onto a carcass from an overhanging object. Clearly there is some confusion regarding the behavior of *N. bullata* and sarcophagids in general.

Other than Mitchell and Soucie (1993) no recent studies of the role of olfaction on larviposition of a sarcophagid have been performed, and their observation was a byproduct of an experiment designed to test another question. To explore this further I used a set of rigid protocols to investigate if olfaction is involved in the inducement of larviposition. The experiments asked two questions: 1) will *N. bullata* deposit without contact chemoreception and 2) will olfaction stimulate a CES for larviposition behavior?

3.2 Materials and Methods

Observations for all experimental trials were conducted between 10:00 am to 6:00 pm, temperature was between 23.5 °C and 24.5°C and the fluorescent lights of the room were left on for all observations. Females tested were raised according to the methods described in Appendix 7.1.

Females were tested in two sequential phases. During phase 1 flies were "preconditioned" to the odor of 67% liver concentrate. Chambers (Appendix 7.2.1) were modified by placing a fine plastic mesh between the plastic cup and the petri dish (Figure 3.1). The petri dishes held a plexiglass receptacle (Appendix 7.2.2) containing a substrate. A fly was placed in the cup out of contact but in close proximity to the substrate (the source of odor). Substrates consisted of two treatments: 1) double deionized water (control) and 2) 67% liver concentrate (treatment). This phase lasted from 66 to 75 minutes.

Phase 2 immediately followed phase 1. Females were transfered to a second unmodified chamber and allowed contact with 67% liver concentrate in a plexiglass receptacle. Phase 2 lasted for 66 minutes. Since no females larviposited during phase 1, all females in phase 2 had no previous larviposition experience.

Times were recorded for two behavioral events during phase 2: 1) the first taste of substrate and 2) the first larviposition event. The Observer software package (Noldus, 1991) was used to record these events. Reaction time of the experimenter had a maximum delay of five seconds from observation to recording of the event. Eleven trials consisting of 64 flies for each treatment group were used. The larvae deposited in the first event were frozen and counted.

Females were used 10-11 days after emergence. In each trial, anywhere from 6 to 18 females were randomly allocated to each treatment for a total of 64 females tested per treatment.

Description and statistical analysis of data were performed using Microsoft Excel (versions 4.0 and 5.0) and SPSS for Windows (version 6.1). G-tests of independence for a Model II design derived from a formula outlined in Sokal and Rohlf (1981) were programmed on Excel worksheets. Williams' correction (Sokal and Rohlf, 1981) was applied for an adjusted G-statistic which was compared to Excel's built in Chi-square distribution for probability values.

All other descriptions, transformations, and statistical analysis (including Shapiro-Wilks and K-S Lilliefors test for normality, Levine statistic for homogeneity, normal and detrended normal Q-Q plots, t-tests of independent samples, and Mann-Whitney U) were performed using SPSS. All statistics will be discussed where appropriate in the Results section.

3.3 Results

3.3.1 Phase 1 (Table 3.1)

Flies given the odor treatment were observed for larviposition. Out of 64 flies given this treatment, none deposited during phase 1. In contrast, 19 of these flies deposited when allowed contact with the substrate during phase 2. Control flies (using water in phase 1) showed similar behavior.

Many flies rested on the sides or tops of chambers for the entire duration of phase 1. Females directly above the substrate made no attempts at tasting nor was any other agitated movement observed. In short, females showed no signs of recognition of the presence of a larviposition substrate or any behavior different from control females. Top portions of chambers contained pungent liver odors when inhaled by the experimenter.

3.3.2 Phase 2

The results of phase 2 were summarized in terms of four response variables. The first two variables were measures of latency until larviposition.

1) Time from the first taste of substrate until larviposition (time from 1st taste) (Table 3.2)

Flies in both treatments usually larviposited quickly after their first taste of substrate (Figure 3.2 and 3.3). The distribution of this interval was heavily skewed to the left for both treatment groups. This skew encompasses the short intervals found by Mitchell and Soucie (1993) for their odor treated group. In these results both treatment groups contained flies with short as well as much longer intervals. In these results, the control group actually had a shorter mean interval than the treated group although this difference was not significant (Mann Whitney U, p=0.8091). Mitchell and Soucie (1993) used shorter trial durations for odor conditioning which might account for the shorter intervals of their odor group.

2) Time from begining of phase 2 until larviposition (time from Go) (Table 3.2)

We might have expected odor treated flies to larviposit more quickly than the controls after the start of phase 2. This was not the case. Both groups of flies were quite variable as to when they would larviposit and latency was fairly evenly distributed during phase 2 (Figure 3.4 and 3.5). The control flies had a lower mean latency; however, this difference was not significant (Mann Whitney U, p=0.1120).

3) Number of larvae and eggs deposited per female during her first event (#larvae/fly/event) (Table 3.2)

The treatments potentially could have altered the numbers of larvae deposited by each fly as was seen in Chapter 2; however no major differences between the groups were seen. The distribution of the #larvae/fly/event was very similar for both treatments, skewed to the left (Figure 3.6 and 3.7). The #larvae/fly/event seen in these trials are also distributed similarily to other trials utilizing the same conditions. One female in the odor treated group deposited an astounding 172 larvae in one event which is the highest value seen in any of the trials performed in this work. This number was considered highly unusual and was not included in the analysis or in the figures. Data were log transformed and a t-test of independent samples (equal variances) was used to test significance. The difference between treatments was not significant (p=0.442).

4) % response

Almost thirty percent of the odor treated flies deposited whereas 25 percent of the control group deposited (Table 3.1). These percentages were analyzed in a 2x2 contingency table and tested using a G-test of independence with a Williams correction. The differences were not significant (p=0.5548).

3.4 Discussion

It is not under debate whether or not flies use olfactory information in oviposition or larviposition behavior. It is well known that the volatile compounds of rotting meat will attract carrion feeding flies from considerable distances. However, the effects of olfaction on inducement of larviposition are far more variable from species to species. For *Phormia regina* and *Lucilia cuprina*, olfaction appears to be the single most important cue in oviposition (Barton Browne, 1960; Barton Browne, 1962). *Cochliomyia hominivorax* still places a great deal of emphasis on olfaction but other cues appear to be highly important modifiers as well (Hammack, 1991).

Olfaction is not necessarily the most important cue for all species. The results presented here support the conclusion of Mitchell and Soucie (1993) that taste is the primary releaser of larviposition. Their suggestion that a state of "preconditioning" or a CES is induced by odors from a suitable substrate was not supported by the present findings. Their results may have been an artifact of an insufficient trial duration. They may have unknowingly selected for flies by chance that larviposited quickly after tasting the substrate. Nevertheless, this only supports the idea that olfaction is relatively unimportant in the inducement of this fly to larviposit compared with taste.

These results conflict with the findings of earlier studies that show sarcophagids and *N. bullata* are stimulated using nothing but odors from a larviposition substrate. This is a long held and widely accepted belief. Wardle (1921), Graenicher (1935), Hallock (1942), James (1947), and Denno and Cothran (1976) either witnessed or suggested that these flies would larviposit in the presence of odors without contact. Some fairly impressive feats of larviposition have been documented including those occurring into bottles (containing rotting beetles) through a tightiy wrapped cheesecloth covering (Hallock, 1942) or on jars with lids spaced to keep flies from entering but to allow odors out (Graenicher, 1935). Many of these studies are fairly old and are often dismissed by suggesting contamination of such materials as cheesecloth and even misindentifications of the species larvipositing.

The reasons for criticizing earlier observations are all valid but no information exists to support these doubts either. Without any contrary evidence the possibility that olfactory information is important to *N. bullata* must be considered. First these observations are common enough to warrant consideration. Second, misidentifications of flesh flies are always possible especially since males are needed to identify species. This is difficult when collecting flies from larviposition sites (Hall and Doisy, 1993). However, *N. bullata* is a well described species known since Parker first described it in 1916. Roback (1954) used it as the type species for the new genus of *Sapromyia*.

It is possible that under different conditions *N. bullata* will larviposit using olfactory information. In a field situation the smell of a carcass might be just as good as the taste since confusion with other substrates seems highly unlikely. Because maggots may develop on many types of carcasses, any substrate that smells about right may be close enough for larviposition. The conditions used in these trials were quite rigid and tested flies only under very specific conditions using fresh as opposed to putrid substrates. Young, healthy females were used which may be quite particular. Tasting in these conditions appears to be crucial for larviposition. Unfortunately no information exists on what conditions may alter the relevance of olfactory stimuli.

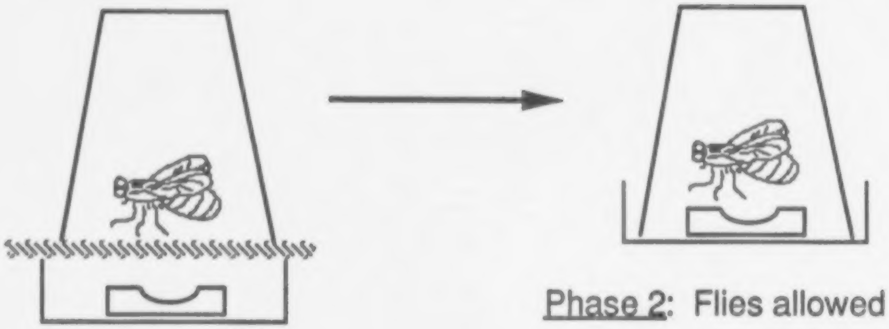
Table 3.1 - Number of flies larvipositing out of contact with the substrate (phase 1 - conditioning) and in contact with the substrate (phase 2). Odor treated flies were conditioned using 67% liver concentrate, control flies with water. Larviposition substrate in phase 2 was 67% liver concentrate. Phase 1 lasted a minimum of 66 minutes; phase 2 lasted 66 minutes.

Treatment	n	phase 1	phase 2
odor	64	0	19 (29.7%)
control	64	0	16 (25.0%)
Total	128	0	35 (27.3%)

Table 3.2 - Comparison of responses on a 67% liver concentrate (phase 2) after conditioning using two treatments (phase 1). Odor treated flies were conditioned using 67% liver concentrate, control flies with water. Phase 2 lasted 66 minutes.

Response variable	treatment	n	mean	min	max	SEM
Time from the 1st taste of substrate until larviposition [a]	odor	18	747.0	2.9	3369.9	259.4
	control	16	644.3	2.1	2386.1	183.1
Time from start of phase 2 until larviposition [b]	odor	19	1859.9	101.6	3398.1	257.1
	control	16	1261.1	104.4	3554.1	266.8
Number of larvae deposited per fly during her first larviposition event [c]	odor	18	11.7	1	43	2.2
	control	15	12.3	1	54	4.0

[a] n.s. (Mann-Whitney U, p=0.8091, Z=-0.2415)
[b] n.s. (Mann-Whitney U, p=0.1120, Z=-1.5894)
[c] n.s. (t-test of independent samples using log transformed data set, p=0.442, t=-0.78, d.f.=31)



Phase 1: Flies conditioned for 1 hour

Phase 2: Flies allowed contact with substrate for 1 hour

Figure 3.1- Apparatus for measuring behavioral responses of females after odor conditioning. During phase 1, odor treated flies were conditioned using 67% liver concentrate, control flies with water. A plastic screen prevents flies from contacting the treatments. After 66 to 75 minutes flies were moved from phase 1 apparatus to phase 2 apparatus for 66 minutes. During phase 2 both treatments are allowed contact with 67% liver concentrate in a plexiglass receptacle.

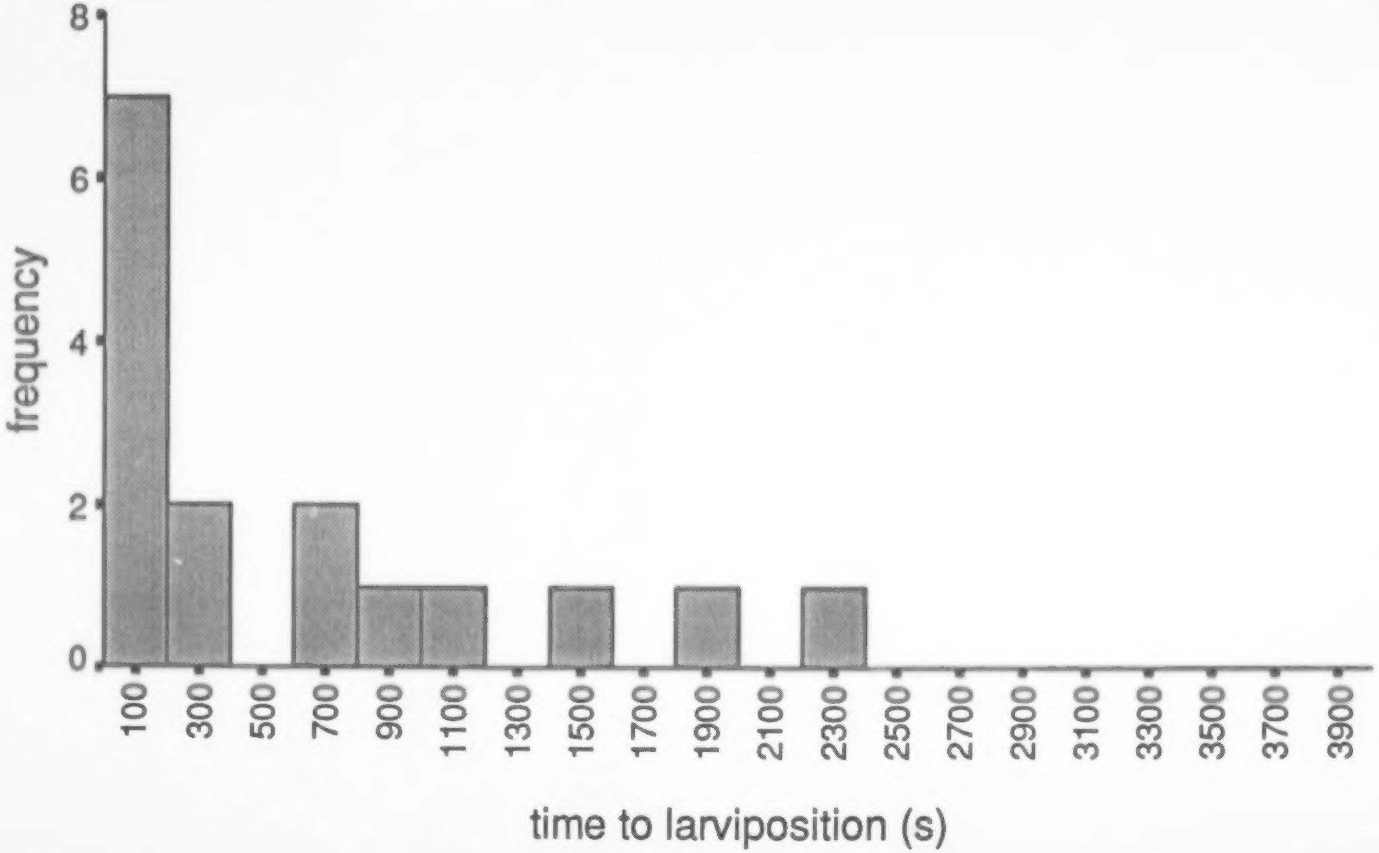


Figure 3.2 - Time to the first larviposition event after the first taste of 67% liver concentrate for control flies during phase 2. Control flies were conditioned using water. Trials lasted 3960 s. Larviposition soon followed the first taste of substrate (mean=644.0, SD=732.44, n=16).

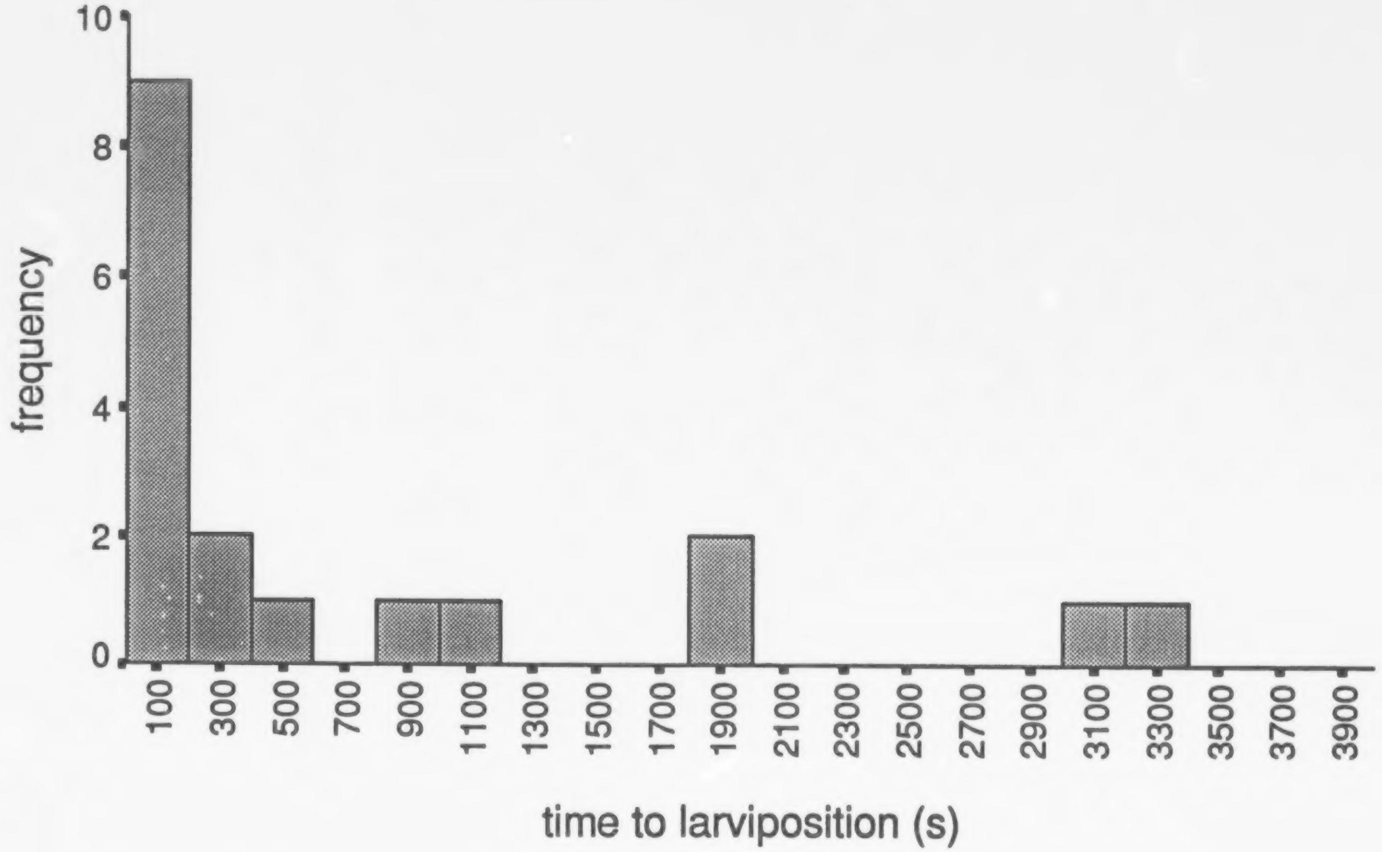


Figure 3.3 - Time to the first larviposition event after the first taste of 67% liver concentrate for odor treated flies during phase 2. Odor treated flies were conditioned using 67% liver concentrate. Trials lasted 3960 s. Larviposition soon followed the first taste of substrate (mean=747.0, SD=1100.75, n=18).

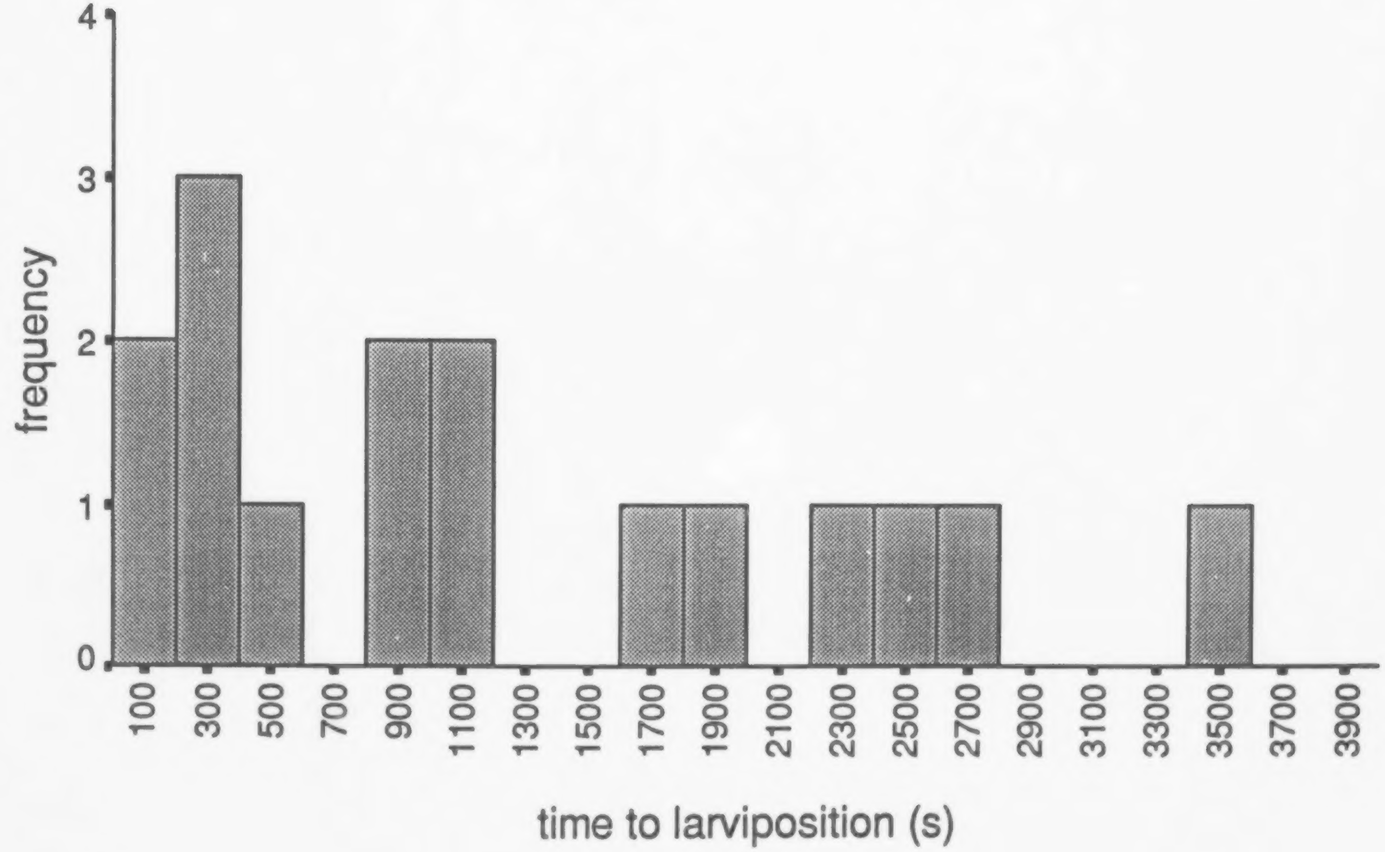


Figure 3.4 - Time to the first larviposition event for control flies during phase 2. Control flies were conditioned using water. Events on histogram refer to the time taken by females to larviposit from the start of phase 2. Trials lasted 3960 s (mean=1261.0, SD=1067.23, n=16).

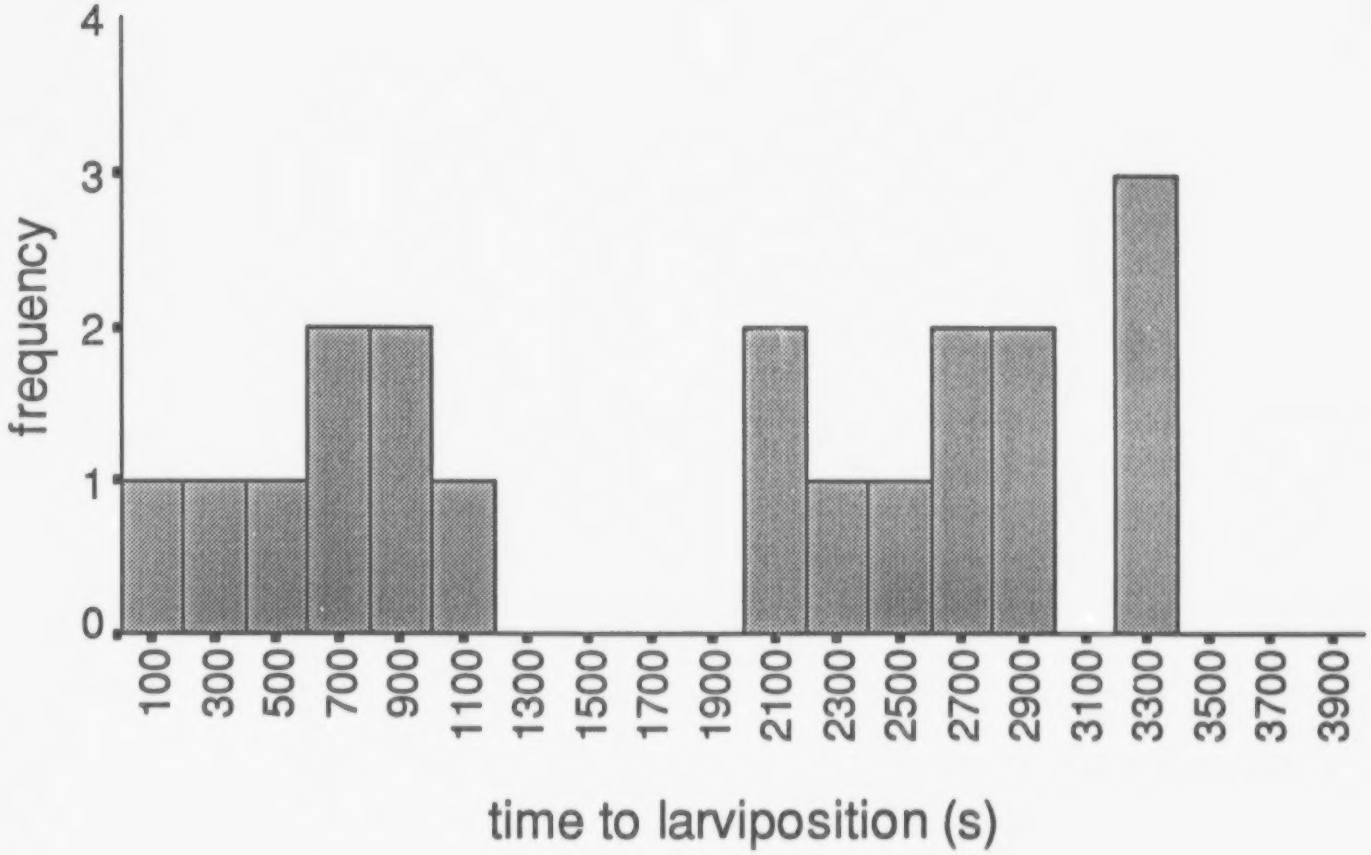


Figure 3.5 - Time to the first larviposition event for odor treated flies during phase 2. Odor treated flies were conditioned using 67% liver concentrate. Events on histogram refer to the time taken by females to larviposit from the start of the phase 2. Trials lasted 3960 s (mean=1860.0, SD=1120.77, n=19).

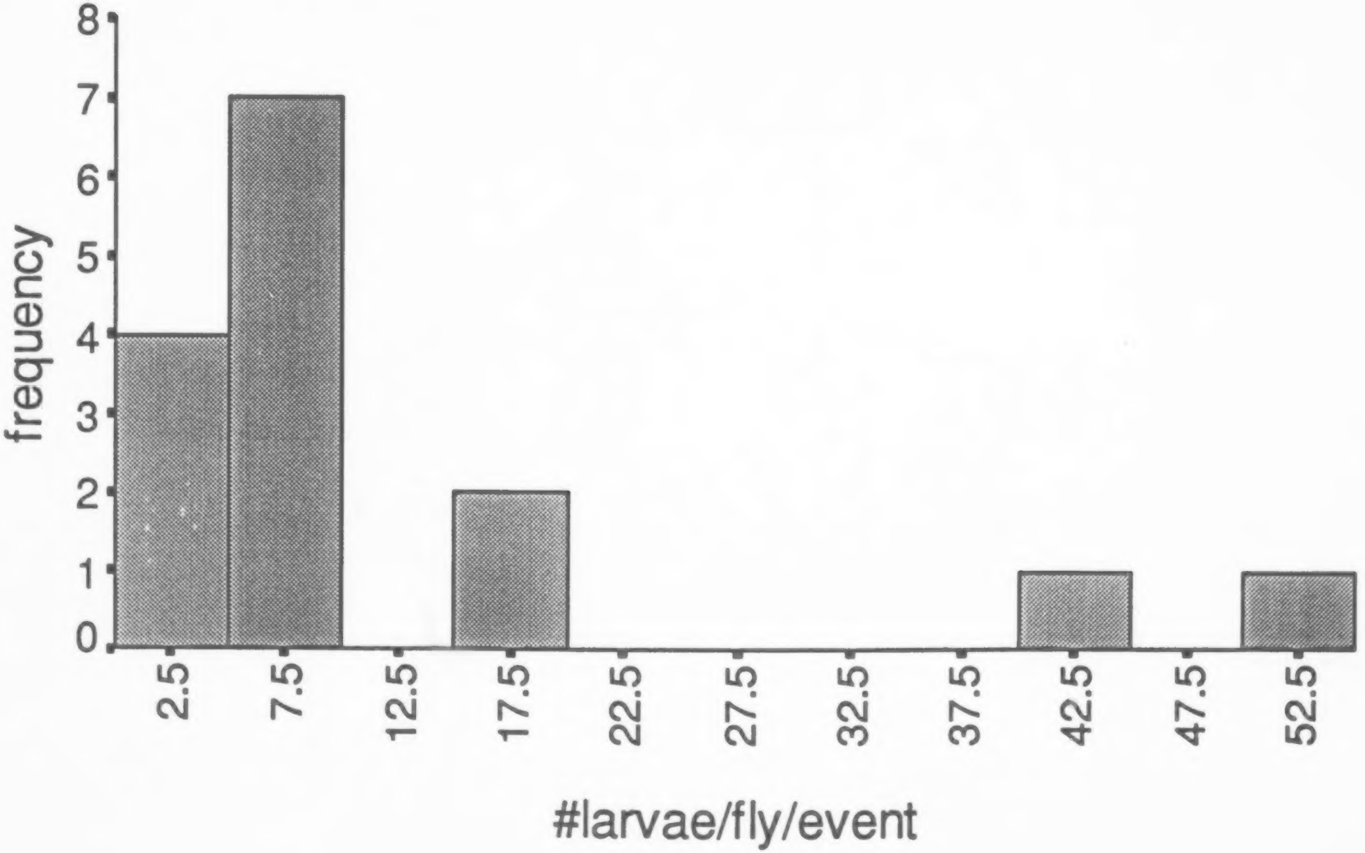


Figure 3.6 - Number of larvae and eggs deposited per female during her first event of larviposition for control flies. Control flies were conditioned using water but used 67% liver concentrate in a plexiglass block as a substrate (mean=12.3, SD=15.55, n=15).

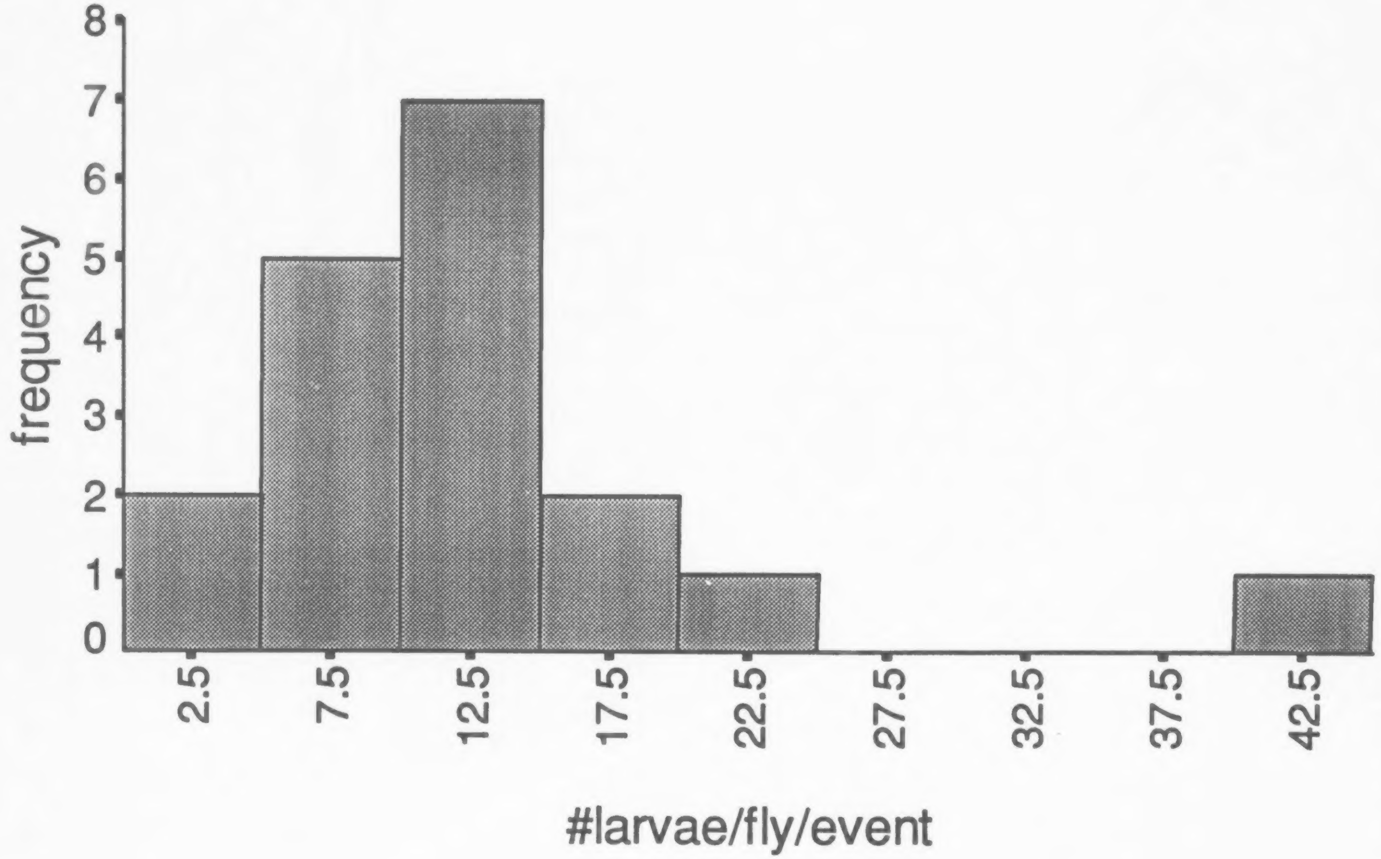


Figure 3.7 - Number of larvae and eggs deposited per female during her first event of larviposition for odor treated flies. Odor treated flies were conditioned using 67% liver concentrate in a plexiglass block and used the same as a substrate (mean=11.7, SD=9.23, n=18).

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Chapter 4 Chemical Analysis of a Larviposition Substrate

4.1 Introduction

Analysis of liver for human nutritional studies are common and extensive. Anderson (1992), Holland et. al. (1992), and McCance and Widdowson (1960) give thorough analyses of raw pork liver focusing on protein, carbohydrate, lipid, mineral, vitamin, and nitrogen or amino acid content. Often these studies include only those portions of liver considered edible or include results for cooked liver. As pointed out by McCance and Widdowson in Holland et. al. (1992) results may vary according to different analytical methods, food preparation methods, or methods of raising animals used over the past 70 years.

For the purposes of this study a liver derivative (Sigma liver concentrate, stock #202-3) was used for the bioassays described in Chapters 2, 3, and 5. Preparation of liver concentrate can be expected to cause changes in chemical properties. Unfortunately the chemical components of liver concentrate have not been analyzed in great detail.

Liver concentrate is prepared for Sigma by a third party and the preparation method is proprietary information. Sigma Technical Service (1-800-325-5832) offers the following information about preparation. Pig livers are ground then subjected to a boiling water extraction. This mixture is cooled and subjected to a high flow-clarified, silicaceous earth extraction and filtered. The resulting mixture is freeze dried.

Sigma Technical Service provided the following information regarding analysis. Liver concentrate contains 5.4% moisture, 8.4% ash residue (ie. minerals, salts, and other noncarbon compounds) on ignition, 211 mg/g riboflavin, 1.2 mg/g nicotinic acid, and 12 mg/g choline. Thin layer chromatography (TLC) that they provided used nucleotide markers and gave the following results: no coenzyme A was present; NAD had a RF between 0.4% and 0.6% and no NADP was present. Their technical service staff cautioned that resolution found by TLC may not have been fine enough to resolve the difference between NAD and NADP.

In order to provide further information on potential chemical stimuli of larviposition behavior, a preliminary analysis of liver concentrate was performed concentrating on protein, amino acid, and nucleotide content. In particular, chemical differences between the liver concentrate and a lab prepared derivative using a chloroform: methanol: water extraction (Bligh and Dyer, 1959) were analyzed.

4.2 Materials and Methods

Liver organs were deemed too difficult to use and standardize in conjunction with long term behavioral bioassays. Therefore, Liver Concentrate from Sigma (stock #202-3, Lot #50H0376) was used as a standardized source of liver constituents for extractions and analysis.

4.2.1 Chloroform: Methanol: Water Extractions

Two methods of extraction were used to remove lipids from liver concentrate. Both are based on the procedure described by Bligh and Dyer (1959). The first was modified from Bielski and Turner (1966). This technique was originally used for separation of small quantities of plant tissues and was ultimately abandoned in this study because only small quantities of material can be processed effectively. The second method was based on the procedure of Woyewoda et. al. (1986b). This technique can be used to process larger quantities of material and was developed for separation of constituents of fish.

4.2.1.1 method a

Four to six grams of liver concentrate were suspended in chloroform: methanol: water (ratio by volume of 12: 5: 3). This mixture was decanted into a Ten Broeck tissue homogenizer and ground for 1 minute. The homogenate was centrifuged in polypropylene centrifuge tubes for

10 minutes at 1310 g measured at the tip of the centrifuge tubes. The supernatant (very dark brown in color) was decanted into a large glass centrifuge tube held on ice.

The residue was resuspended in C:M:W, then transferred to the tissue homogenizer and reground for 1 minute. The homogenate was centrifuged in polypropylene centrifuge tubes for 6 minutes at 1310 g measured at the tip of the centrifuge tubes. Again the supernatant was decanted into the large glass centrifuge tube held on ice. This process was repeated 3 to 4 more times until the resulting supernatant was a translucent, pale yellow color. The remaining residue was suspended in ca. 3 ml of water and transferred to a preweighed, aluminum weigh boat to dry at room temperature until no liquid remained. The dried insoluble material was later weighed.

Equal volumes of double distilled, deionized water and chloroform were added to the supernatant which was centrifuged to enhance phase separation. This mixture separated into two distinct phases separated by a thin white layer of particulate material. The upper phase (methanol: water soluble) material was very dark brown whereas the lower phase (chloroform: lipid soluble) material was pale yellow in color.

The upper phase was transferred by pasteur pipette to a round bottomed flask. The remaining supernatant was washed three times with small volumes of water to maximize recovery and the remaining upper phase transferred after each wash. The lower phase was washed with a small volume of chloroform and then transferred to a round bottomed flask. Most of the particulate matter was left behind.

A rotary evaporator was used to dry off the methanol in the upper phase and to reduce the volume of water. The temperature was kept between 30 to 40 ^{o}C using a water bath. The volume of the upper phase was reduced by over 75% within 2 to 3 hours. Evaporation continued until viscosity visibly increased.

The same setup was used for the lower phase. All the solvent could be removed within 30 to 45 minutes. This left behind a yellow, oily film on the round bottomed flask.

The water soluble material was transferred to two preweighed, glass freeze dryer flasks. The contents were frozen in a dry ice/70% ethanol bath and placed on a freeze dryer (Virtis 10-145 MR-BA Freeze-mobile) overnight. The pressure was kept at < 1mm Hg and the operating temperature was between -40 to -45^{o}C. The resulting water soluble powder was weighed in the freeze dryer flasks on a Sartorius scale. The powder removed from the flasks was weighed again on a Mettler College before being mixed with water for use in behavioral assays.

All equipment used was weighed before use. Equipment was allowed to dry after use and reweighed where possible to obtain mass of constituents and account for losses of material. Four separate extractions were performed using this method.

4.2.1.2 method b

Lipids were extracted from water soluble constituents using a slightly modified version of method B described by Woyewoda et. al. (1986b). ACS grade chloroform (50 ml), ACS grade methanol (100 ml), and double distilled, deionized water (40 ml) were added to 10 to 15 grams of liver concentrate. This mixture was blended using a Waring blender at high speed for 3 minutes. 50 ml of chloroform was added and blended again for 1 minute. 50 ml of water was added and blended again for 1 minute.

In the first three blending steps chloroform, methanol, and water are maintained in ratios of 1:2:0.8, 2:2:0.8, and 2:2:1.8, the last ratio creating a biphasic mixture. Due to the large quantity of water soluble constituents present it was desirable to further enhance the separation of the two phases, therefore another 50ml of water was added and blended for an additional minute. This results in a final ratio of 2:2:2.8, still w[illegible]nder the "maximum chloroform tie-line" of Bligh and Dyer (1959).

The mixture was filtered through a preweighed Whatman #4 filter paper using a Buchner funnel and water aspirator. All equipment was rinsed with chloroform/ methanol (1:1). Insoluble material collected on filter paper was allowed to dry and reweighed. The mixture was transferred to a 1000 ml separatory funnel and allowed to sit for a minimum of 2.5 hours.

A cone was formed using #4 filter paper folded inside a #1 filter paper and filled with granular sodium sulfate (ACS grade). The lower chloroform layer was filtered through the cone into a preweighed round bottomed flask then refrigerated at 4°C. A small portion (< 1 ml) of the remaining upper methanol: water layer was drained off and the rest was drained into two preweighed round bottomed flasks (total volume >500 ml).

Due to the large volume of solvent present in the methanol: water flasks freeze drying could not be accomplished without using a rotary evaporator first. This usually could not be accomplished in one day and contents were chilled at 4°C overnight. Rotary evaporation was accomplished using temperatures between 30°C to 36°C for a duration of 4 to 6 hours per flask, pressure could not be measured. Evaporation continued until a noticeable increase in viscosity of the mixture occurred. The same procedure was used for evaporation of solvent from the lipids, although only 30 to 45 minutes was required before lipids gelled in their container.

Water soluble mixtures were transferred to two preweighed glass freeze dryer flasks. Contents were frozen in a dry ice/70% ethanol bath and placed on a freeze dryer (Virtis 10-145 MR-BA Freeze-mobile) overnight. Pressure was kept at < 1mm Hg and temperature was maintained between -40 to -45°C. The resulting water soluble powder was weighed in the freeze dryer flasks and again before being mixed with water for use in behavioral assays. Equipment was allowed to dry and reweighed where possible to obtain constituent masses and account for losses of material. Three separate extractions were performed using this method.

4.2.2 Analysis of constituents

An attempt to find differences in chemical constituents between liver concentrate and the derived water soluble fraction was undertaken. The water soluble fraction from one extraction using method Ib and liver concentrate were subjected to the analyses outlined below.

4.2.2.1 Amino Acid Analysis using HPLC

Both types of liver fraction, liver concentrate and the water soluble constituents isolated in section 4.2.1.2 were analyzed for amino acid content. Free amino acids as well as amino acids released during hydrolysis were measured. Amino acid content was determined using high performance liquid chromatography according to the method in Sedgwick et. al., (1991). 25 µl of mixed samples were separated and quantified using a Varian 5000 high performance liquid chromatograph and Varian Fluorichrom detector.

a) Free amino acid samples were prepared by diluting about 50 mg of either fraction with 3.0 ml of H_2O. About 0.05 ml of these solutions were mixed with 0.1 ml of 25 nmol/ml BABA/EA (ß-amino-butyric acid/ ethanolamine), 0.2 ml of saturated $K_2B_4O_7$, and 1.0 ml of H_2O. Samples were centrifuged at 3000 RPM for 5 minutes.

An amino acid standard solution was prepared by mixing Sigma Amino Acid Solution, glutamine, asparagine, tryptophan, citrulline, taurine, and ornithine for a final concentration of 25 nmol/l. The standard was mixed with 0.1 ml of BABA/EA, 0.2 ml $K_2B_4O_7$, and 1.05 ml of H_20.

b) Hydrolysates of both fractions were prepared as follows to release amino acids bound in macromolecules. About 64 mg of both fractions were hydrolyzed with 5 ml of 6M HCl for 24 hours at 110°C. Then 0.025 ml of samples were mixed with 0.2 ml of 25mmol/ml BABA/EA, 1.4 ml H_20, 0.026 ml of 5.29 M NaOH, and 0.2 ml of $K_2B_4O_7$. Samples were centrifuged for about 5 minutes at 3000 rpm.

Standard solutions were prepared by mixing 0.2 ml of 25 nmol/L Amino Acid Standard (Sigma Amino Acid Solution, glutamine, asparagine, tryptophan, citrulline, taurine, and ornithine), 5 ml of 6M HCl, 0.2 ml of 25 mmol/ml BABA/EA, 1.2 ml H_20, 0.026 ml of 5.29 M NaOH, and 0.2 ml of $K_2B_4O_7$.

Analysis was performed on two replicates of free amino acid preparations, two of the hydrolysate of liver concentrate, and three of the hydrolysate of the water soluble fraction. Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography system. Final data analysis was performed using Excel 4.0.

4.2.2.2 Protein Analysis

a) Capillary SDS Gel Electrophoresis

Proteins that occur in the 14 200 Da to 205 000 Da molecular weight range were analyzed using the eCAP SDS 14-200 capillary electrophoresis size separation kit (Beckman part no. 477420) and outlined procedures. Samples were introduced using pressure injection (60 sec.) into a 75 µm internal diameter, 57 cm long (50 cm to detector window), fused silica capillary. Temperature of the capillary was maintained at 25^oC and the inlet was held at 10 kV after injection. The capillary was sequentially washed with 1.0 N hydrochloric acid then reconditioned with SDS 14-200 gel buffer. On column UV absorbance detection was used at 214 nm with a 100X200 µm aperture in a Beckman P/ACE cartridge. Analysis was performed using a Beckman P/ACE System 2100 in reversed polarity mode, controlled by a Prospect 486 computer. Post-run data analysis was performed using Beckman System Gold Version 8.1.0 software.

Protein concentration was determined using the Lowry et. al. (1951) method. Protein extractions were performed using 1:10 phosphate buffer saline (PBS) at pH 6.9. Test samples were prepared according to Beckman's SDS 14-200 Kit (1993).

The Beckman SDS 14-200 Test mix included the following protein standards: α-Lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), ß-galactosidase (116 kDa), and myosin (205 kDa). Orange G (10µl) was an internal standard. Standards were prepared according to Beckman's SDS 14-200 Kit (1993).

b) SDS PAGE (polyacrylamide gel electrophoresis) on a slab gel

Samples of both fractions were run on a 10% polyacrylamide gel with SDS according to Laemmli (1970). Samples were incubated 2:1 (v/v) with tracking dye. Final concentration for liver concentrate was 24.9 µg/µl and for the water soluble fraction 39.3 µg/µl. The liver concentrate was placed in wells containing 3, 6, and 15 µl quantities corresponding to 74.7, 149.4, and 373.5 µg of sample. The water soluble fraction was placed in wells containing 2, 4, 10, and 15 µl quantities corresponding to 78.6, 157.2, 393.0, and 589.5 µg of sample.

A BioRad broad molecular weight standard was used to identify the molecular weights of the protein bands. The composition of the BioRad broad molecular weight standard was myosin (200 kDa), ß-galactosidase (116.5 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). The standard was placed in a well containing 5 µl which corresponds to 0.5 µg of protein standard per band.

4.2.2.3 Nucleotide Comparison using Micellar Electrokinetic Capillary Chromatography (MECC)

Nucleotides were extracted from liver concentrate and its water soluble fraction by mixing 1:2 (w/v) with 6% (w/v) $HClO_4$ and neutralized with 25% (w/v) KOH (Woyewoda et. al., 1986a). Solutions were diluted 1:2 (v/v) with double distilled, deionized water. Electrokinetic injection (5 kV, 5 sec.) was used with a 75 µm internal diameter, 57 cm long (50 cm to detector window), fused silica capillary. Temperature of the capillary was maintained at 25^{o}C and the inlet was held at 12.5 kV after the electrokinetic injection. The capillary was sequentially washed with 1N sodium hydroxide and rinsed with the buffer system between runs. On column UV absorbance detection was used at 260 nm with a 50 x 200 µm aperture in a Beckman P/ACE cartridge. Analysis was performed using a Beckman P/ACE System 2100 set with the anode at the detector end of the capillary, controlled by a Prospect 486 computer. Post-run data analysis was performed using Beckman System Gold Version 8.1.0 software. Phosphate buffer (pH 7.0) consisted of 50 mM sodium phosphate (mono-/dibasic sodium phosphate), 100 mM DTAB (dodecyl trimethyl-ammonium bromide), and 1 mM EDTA (ethylenediaminetetracetic acid) was prepared using double distilled, deionized water. Standards consisted of 50 µM nucleotides (ATP, ADP, AMP, and IMP) and nucleosides (HxR, Hx, and XAN) at concentrations of 25 µM, 1.15 µM, and 10 µM respectively.

4.3 Results

4.3.1 Results of C:M:W extraction, quantitative measurements

Regardless of the extraction method, relative proportions of constituents by weight stayed approximately the same for liver concentrate and the water soluble fraction (Table 4.1). Water soluble constituents were present in the largest quantities, roughly 93 to 94 %. The value of 95.9% is probably somewhat high and the result of measurement error since over 100% recovery was obtained for that extraction. Insoluble material was consistently present in quantities of roughly 4 to 5%. Lipids are far more difficult to measure accurately or consistently since they are present in such small quantities.

Qualitative physical properties of the fractions were similar but not exactly the same. Both appeared very dark brown to almost black in color. Settling of material was never observed in either fraction once properly mixed. Liver concentrate appears slightly more viscous than the water soluble fraction when poured. Liver concentrate foams readily when stirred, almost a quarter of a solution can be made to foam when stirred vigorously. The water soluble fraction does not foam and only forms a few bubbles when stirred.

4.3.2 Amino Acid analysis using HPLC

4.3.2.1 Free amino acids

No major differences were apparent in the free amino acids between liver concentrate and its water soluble fraction (Figure 4.1 and Table 4.2) and the conclusions made herein apply to both. Seventeen of the twenty most common amino acids in biological organisms were found with the exceptions of cysteine, glutamine, and proline. Cysteine is not be detected by this method without converting to cysteic acid first (Cunico and Schlabach, 1983; Jones and Gilligan, 1983). Like most secondary amino acids, proline is not detected using o-phthaldialdehyde (OPA) without the addition of sodium hypochlorite (Cunico and Schlabach, 1983). The absence of glutamine is somewhat unusual but this compound probably was destroyed during preparation of the liver concentrate.

Three other compounds appeared in this analysis: citrulline, ornithine, and taurine. These are not found in proteins and therefore are more likely to be detected as part of the free amino acid content of liver. Citrulline and ornithine are both intermediates in the synthesis of urea, a metabolic pathway related to liver function. These two amino acids are not found in proteins (Voet and Voet, 1990). Taurine is not an amino acid but like glycine combines with cholic acid to form bile salts (Mathews and van Holde, 1990).

Free amino acids were not present in large quantities but totalled ca. 13% of each type of fraction by weight. Leucine was present in the largest amount, ca. 1.7% of the sample, whereas all other compounds comprised <1.5%. Arginine, citrulline, and taurine were present in very small quantities, <0.15% of the sample.

Flies are likely to taste free amino acids in a solution as opposed to those bound in a protein moiety. Concentrations of free amino acids in a 67% solution were calculated in Table 4.3

for both fractions. All amino acids are present in solution at concentrations of less than 0.1 M. Alanine and leucine were closest to 0.1 M at about 0.09 and 0.08 M, respectively.

4.3.2.2 total amino acids (free amino acids and amino acids released by hydrolysis)

Figure 4.2 and Table 4.4 show total amino acids comprising free amino acids and those released during hydrolysis of macromolecules. Both fractions show similar totals but hydrolysis clearly releases a great quantity of the amino acids bound in the macromolecular structure compared with those seen in a free state. Total amino acids composed over 50% of each fraction, which reflects the relative abundance of macromolecules.

Fifteen of the most common amino acids were present with the exceptions of: asparagine, cysteine, glutamine, proline, and tryptophan. Asparagine and glutamine are hydrolyzed into aspartic and glutamic acids. Proline can not be detected using OPA (Cunico and Schlabach, 1983). Tryptophan is destroyed during hydrolysis (Friedman et. al., 1984). Cysteine is not be detected without converting to cysteic acid first (Cunico and Schlabach, 1983; Jones and Gilligan, 1983). Citrulline, ornithine, and taurine, which are not components of macromolecules, are also destroyed by hydrolysis (Turchinsky, pers. com.).

Hydrolysis time can affect the quantity of amino acids recovered (Happich, 1981; Rowan et. al., 1992) and therefore only general conclusions can be made about relative quantities in liver concentrate. Glutamic acid was present in the largest quantity (8.4%). However, since glutamine is hydrolyzed to form glutamic acid it is not known how much of this is hydrolyzed glutamine. Aspartic acid constituted a large proportion of the amino acids but it is not certain how much of this is hydrolyzed asparagine. Leucine was the next most abundant amino acid at nearly 5% of the sample. Histidine and methionine were both present at <1.5% of the sample, the least abundant amino acids in the mixture analyzed. However, this method is not quantitative for methionine and the accuracy of this value is questionable (Happich et. al., 1981; Sedgwick, pers. com.).

4.3.3 Protein analysis

Capillary SDS gel electrophoresis and slab SDS PAGE gave slightly different results regarding protein content although it was an almost undetectable difference. No proteins were detected between about 11 kDa and 230 kDa in either fraction using capillary electrophoresis (Table 4.5). Identification of compounds outside this range are unreliable since the standards only encompassed 14.2 kDa to 205 kDa. Four high molecular weight compounds were detected between approximately 230 kDa and 580 kDa and seven lower molecular weight compounds were detected between 1.3 kDa and 11 kDa for both fractions. Figure 4.3 shows the CE SDS-gel profiles for both fractions to be very similar.

Slab SDS PAGE (Figure 4.4) showed a slight difference between the fractions. Very small amounts of protein were detected in liver concentrate around 50 kDa with smaller amounts present around 44 kDa. These were only apparent when using 15 µl (373.5 µg) of fraction. These bands were not apparent in the water soluble fraction even when 15 µl (589.5 µg) of fraction were loaded on the slab gel. Liver concentrate also resulted in faintly colored streaks on the gel which were far less apparent using the water soluble fraction. These streaks are believed to be trace amounts of protein. Bands below 14.4 kDa did not separate well on 10% SDS PAGE using the Laemmli (1970) system and heavy molecular weight bands are apparent in both fractions. These indicate large amounts of small molecular weight peptides and probably correspond with those found using capillary SDS gel electrophoresis.

4.3.4 Nucleotide analysis using micellar electrokinetic capillary chromatography

Due to the complexity of the nucleotide profile of the liver concentrate (Figure 4.5) specific nucleotides could not be identified. Each peak was fairly broad and indicates the presence of nucleotides not resolved using the technique. These nucleotides could probably be identified although time did not permit this during this study. However, nucleotide profiles (Figure 4.5) show a high degree of similarity between liver concentrate and its water soluble fraction.

4.4 Discussion

Liver concentrate is primarily composed of water soluble components. Literature values for the lipid content of raw pork liver range from 6.8 to 7.6% (Anderson, 1992; Holland et. al., 1992; McCance and Widdowson, 1960), it is obvious the preparation of liver concentrate removes a great deal of lipid. Some insoluble material still remains probably composed of structural material such as collagen and cell membranes. It is not known how much of this material might be protein that precipitated during chloroform: methanol: water extraction. It is also not known how much volatile material was extracted during rotary evaporation. It is assumed to be a small amount since recovery of material from liver concentrate was never less than 97%. Both the water soluble and lipid fractions had very pungent aromas present.

Differences between the water soluble fraction and liver concentrate were slight in terms of water soluble components. Liver concentrate shows small amounts of protein in the molecular weight range of 44 and 50 kDa not evident in the water soluble fraction. However, this difference was not evident until large quantities of protein were loaded onto a thin slab gel. Some protein may have been lost during the chloroform: methanol extraction due to solubility changes. This difference was not reflected in the amino acid content of either the free amino acids or those released from the protein during hydrolysis. The nucleotide profile of both fractions was complex and difficult to separate for identification of components. However, profiles of components for both fractions are similar in appearance and it is not likely any major differences exist in either material. On this basis, it is concluded that extracting the lipid and insoluble components of liver concentrate made little difference to the water soluble makeup of the isolated water soluble fraction.

Caution should be used when comparing these figures with established values in nutrient composition tables (Anderson, 1992; Holland et. al., 1992; McCance and Widdowson, 1960). Only crude comparisons with Anderson (1992), Holland et. al. (1992), and McCance and

Widdowson (1960) are possible because the starting material and methods of preparation differ greatly. Since nutritional studies are concerned with human nutritional concerns and not chemosensory stimuli of flies this should be expected.

For future analysis, more detailed comparisons might be appropriate. Proteins are a useful indicator of changes between extracted fractions although the relevance of large molecules to chemosensory stimuli of flesh flies is not particularly well studied. Studies of crustaceans suggest high molecular weight fractions of over 10 000 Da are not effective stimulants (Ache et. al., 1976; Johnson and Ache, 1978; Zimmer-Faust et. al., 1984). The importance of amino acids to many organisms is well known but none of these have been shown for oviposition or larviposition in flies. Nucleotides such as ATP have been shown to be important feeding stimulants for crustaceans and blood feeding insects (Ascoli-Christensen et. al., 1991; Carr, W. E. S. et. al., 1986; Friend, 1978; Friend and Smith, 1975; Friend and Smith, 1977; Friend and Smith, 1982; Friend and Stoffolano, 1984; Mitchell, 1976a; Mitchell, 1976b; Zimmer-Faust et. al., 1988; Zimmer-Faust, 1987). ATP has even been shown to have significant effects on the action of chemosensory cells in neurophysiological studies of a carrion feeder, *Phormia regina* (Liscia, 1985; Liscia, et. al., 1987; Liscia et.al., 1995). Even very simple compounds such as inorganic salts are well known for effects on feeding behavior but were not analyzed in the present study. Future analysis might want to concentrate more heavily on nucleotide analysis and salt content.

Table 4.1 - Composition of Sigma liver concentrate determined using chloroform: methanol: water extraction.

Fraction	n	mean (%)	SEM	minimum (%)	maximum (%)
water soluble	6	94.1	0.49	92.3	95.9
lipid	6	1.1	0.35	0.2	2.4
insoluble	7	4.1	0.14	3.8	5.0

Table 4.2 - Free amino acids in Sigma liver concentrate and in its extracted water soluble fraction as determined using HPLC. Amino acid contents are described as a percentage of the starting mass of either fraction.

Amino acid	liver concentrate (% of sample)	SEM	water soluble fraction (% of sample)	SEM
alanine	1.237	0.052	1.223	0.045
arginine	0.112	0.006	0.107	0.016
asparagine	0.775	0.024	0.744	0.030
aspartic acid	0.622	0.021	0.587	0.019
citrulline	0.036	0.018	0.038	0.022
glutamic acid	0.728	0.025	0.691	0.027
glutamine				
glycine	0.618	0.020	0.621	0.031
histidine	0.297	0.009	0.276	0.009
isoleucine	0.733	0.028	0.754	0.038
leucine	1.616	0.070	1.680	0.090
lysine	1.104	0.052	1.068	0.069
methionine	0.381	0.015	0.378	0.017
ornithine	0.873	0.047	0.859	0.039
phenylalanine	0.802	0.028	0.801	0.034
serine	0.656	0.015	0.640	0.024
taurine	0.112	0.006	0.102	0.000
threonine	0.711	0.013	0.704	0.054
tryptophan	0.277	0.011	0.251	0.012
tyrosine	0.683	0.029	0.602	0.002
valine	1.083	0.037	1.080	0.049
Total	13.452		13.202	

Table 4.3 - Calculated concentrations of free amino acids in 67% liver concentrate or equivalent dilution of its derived water soluble fraction.

Amino acid	liver concentrate (M)	water soluble fraction (M)
alanine	0.093	0.091
arginine	0.004	0.004
asparagine	0.039	0.038
aspartic acid	0.031	0.029
citrulline	0.001	0.001
glutamic acid	0.033	0.031
glutamine		
glycine	0.055	0.055
histidine	0.013	0.012
isoleucine	0.037	0.038
leucine	0.082	0.085
lysine	0.050	0.049
methionine	0.017	0.017
ornithine	0.044	0.043
phenylalanine	0.032	0.032
serine	0.042	0.041
taurine	0.006	0.005
threonine	0.040	0.039
tryptophan	0.009	0.008
tyrosine	0.025	0.022
valine	0.062	0.061

Table 4.4 - Total amino acids present in Sigma liver concentrate and its extracted water soluble fraction determined using HPLC. Total amino acids include free amino acids and those released by hydrolysis of fractions. Amino acid contents are described as a percentage of the starting mass of either fraction.

Amino acid	liver concentrate (% of sample)	SE	water soluble fraction (% of sample)	SE
alanine	4.387	0.043	4.764	0.392
arginine	2.178	0.030	1.924	0.098
asparagine				
aspartic acid	5.089	0.096	5.228	0.417
citrulline				
glutamic acid	8.414	0.024	8.636	0.721
glutamine				
glycine	4.636	0.019	5.077	0.413
histidine	1.333	0.001	1.446	0.105
isoleucine	2.340	0.036	2.655	0.216
leucine	4.987	0.157	5.725	0.525
lysine	4.566	0.194	5.155	0.437
methionine	1.256	0.033	1.403	0.127
ornithine				
phenylalanine	2.557	0.076	2.807	0.232
serine	2.586	0.058	2.717	0.223
taurine				
threonine	2.830	0.043	2.785	0.225
tryptophan				
tyrosine	1.951	0.074	2.051	0.140
valine	3.527	0.102	3.848	0.350
Total	52.634		56.220	

Table 4.5 - Macromolecules detected using capillary gel electrophoresis. Molecules detected are outside range of protein standards (14 400 Da to 230 000 Da). Molecules are labelled by migration time and computer identification of molecular weight.

liver concentrate	
migration time (minutes)	**molecular weight (Da)**
12.73	1379.2
13.94	2905.4
14.51	3969.4
14.93	4901.4
15.75	7198.3
16.13	8507.8
16.71	10770.9
31.62	238954.9
32.69	267710.0
40.98	528514.8
42.37	577362.2

water soluble fraction	
migration time (minutes)	**molecular weight (Da)**
12.73	1375.3
13.93	2903.4
14.26	3482.9
14.96	4980.8
15.75	7191.0
16.15	8569.4
16.22	8821.5
31.59	238188.5
32.65	266715.2
41.02	529992.0
42.40	578228.4

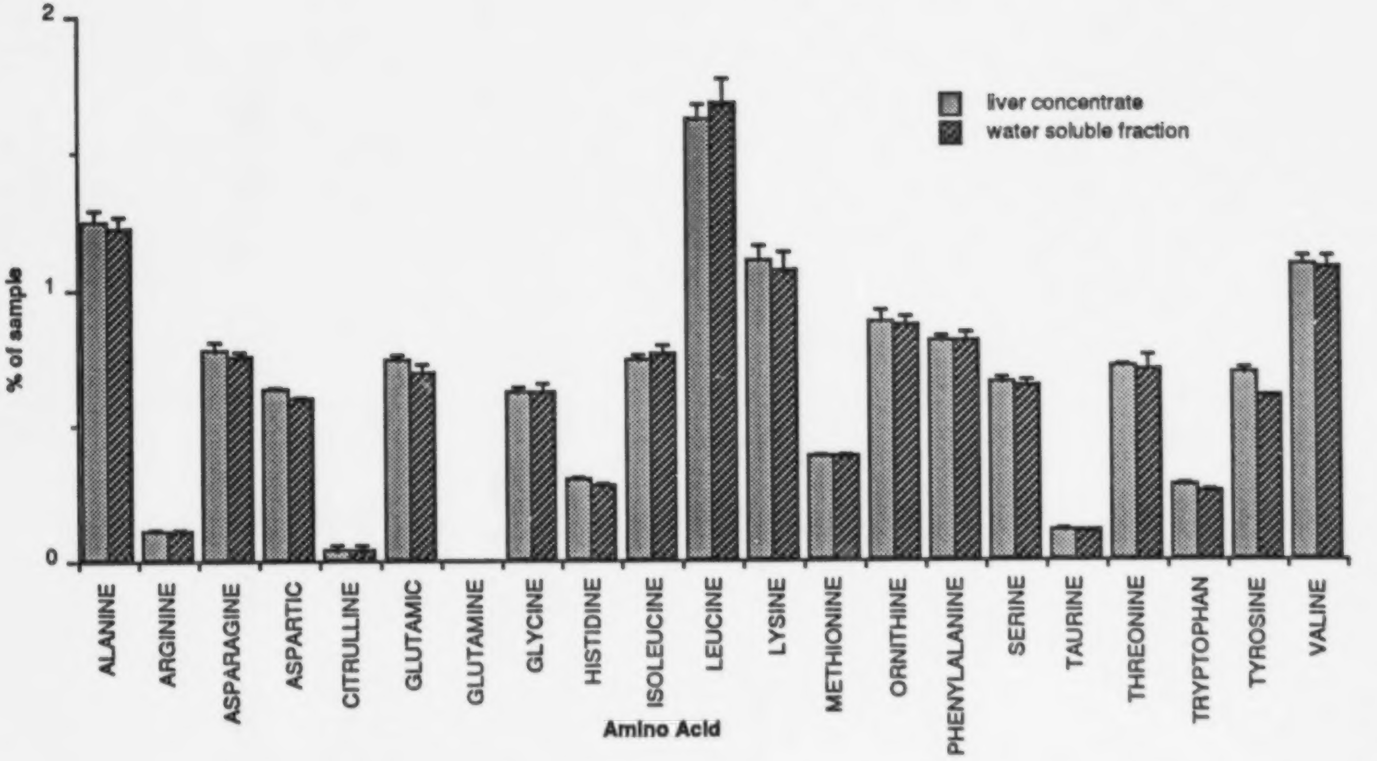

Figure 4.1 - Free amino acids in Sigma liver concentrate and in its extracted water soluble fraction as determined using HPLC. Amino acid contents are shown as a percentage of the starting mass of either fraction. Error bars represent one SEM (n=2).

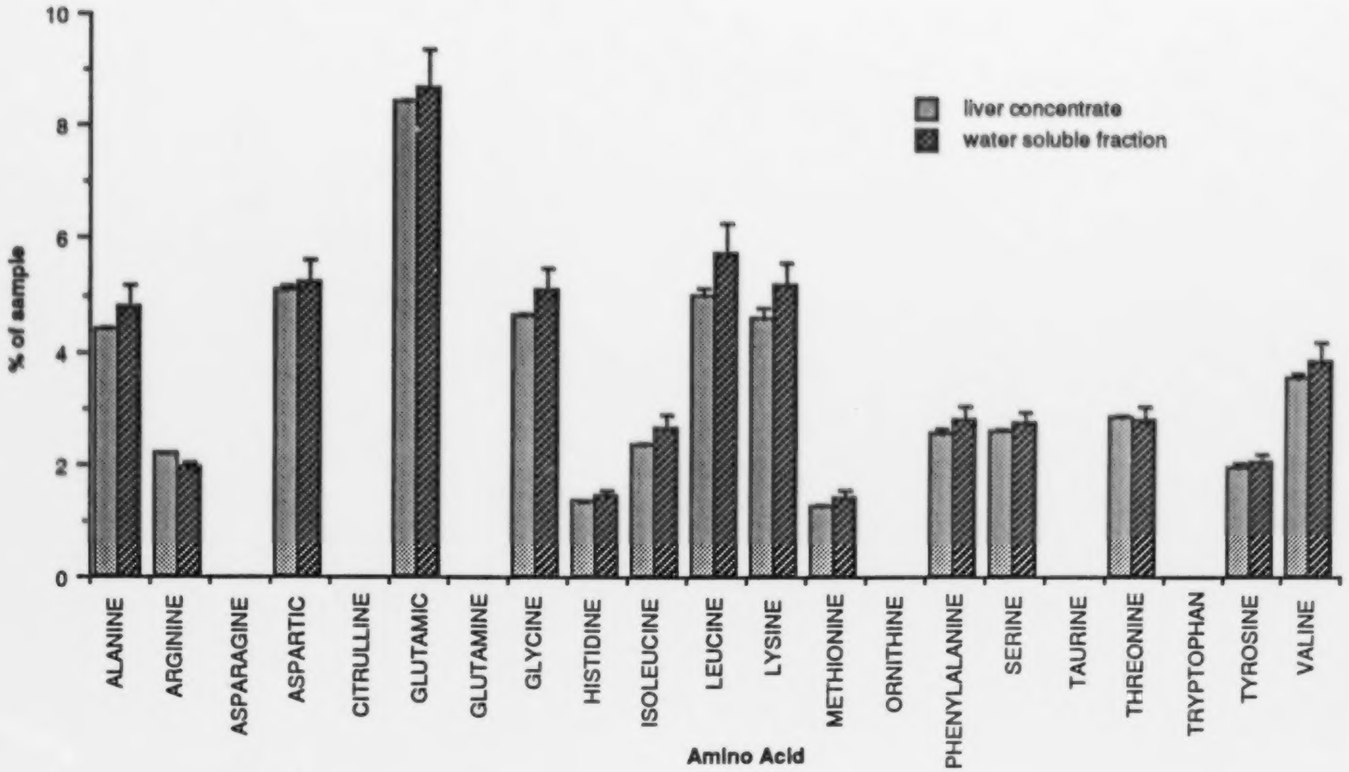

Figure 4.2 - Total amino acids present in Sigma liver concentrate and in its extracted water soluble fraction as determined using HPLC. Total amino acids include free amino acids and those released by hydrolysis of fractions. Amino acid contents are shown as a percentage of the starting mass of either fraction. Error bars represent one SEM (n=2 for liver concentrate, n=3 for its water soluble fraction).

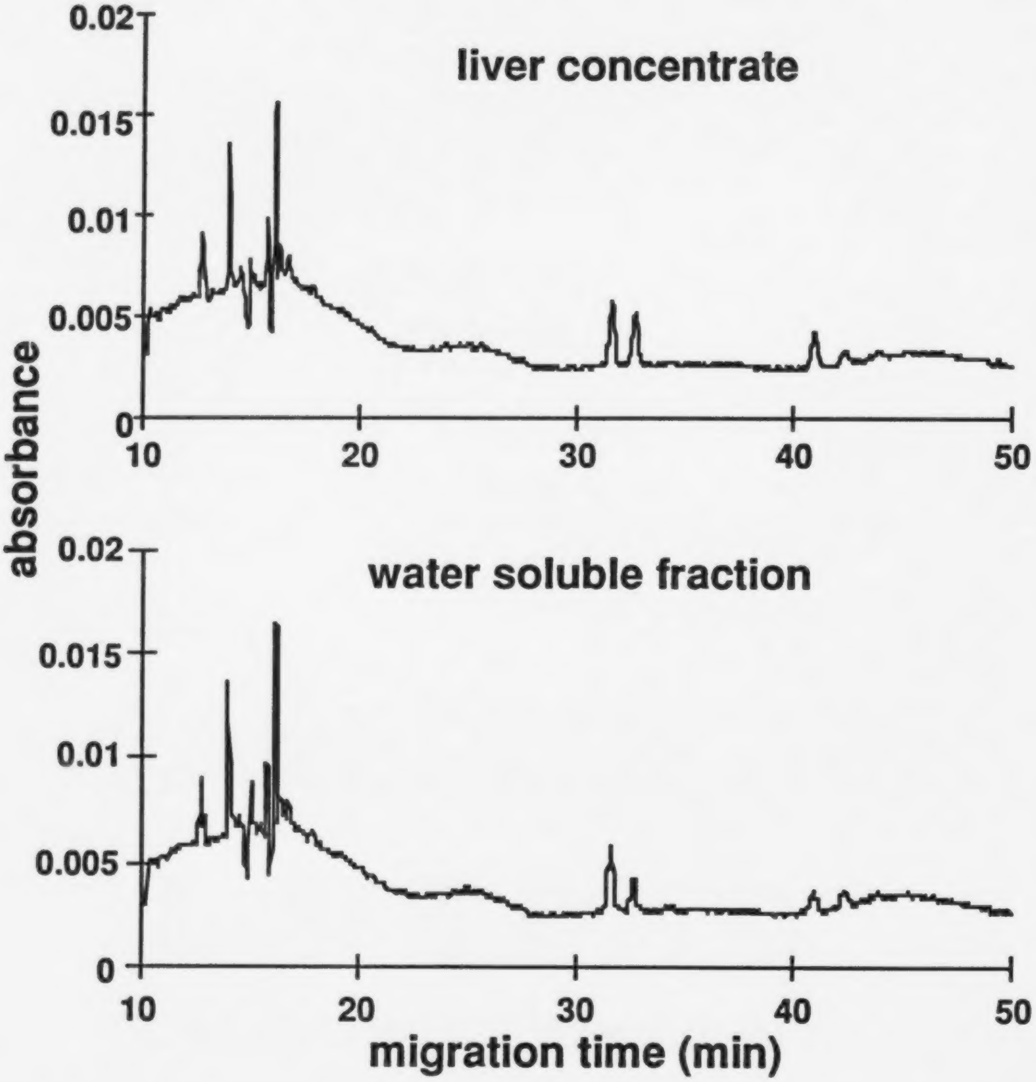

Figure 4.3 - Comparison of protein content of Sigma liver concentrate and in its extracted water soluble fraction using capillary gel electrophoresis. Peaks are outside the range of protein standards (14 400 Da to 230 000 Da).

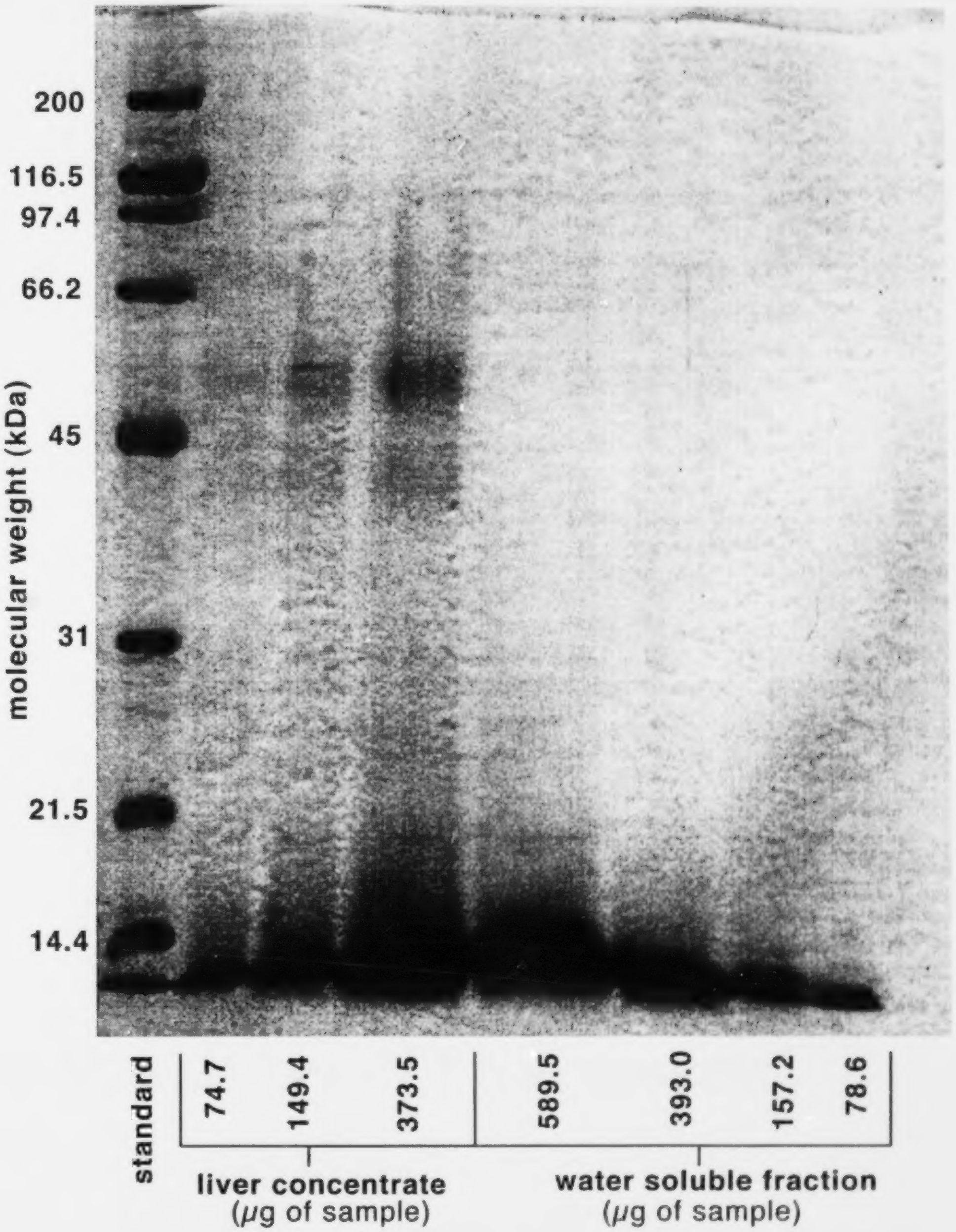
molecular weight (kDa)
200
116.5
97.4
66.2
45
31
21.5
14.4
standard
74.7
149.4
373.5
589.5
393.0
157.2
78.6
liver concentrate
(μg of sample)
water soluble fraction
(μg of sample)

Figure 4.4 - Comparison of protein content of Sigma liver concentrate and its extracted water soluble fraction using SDS-polyacrylamide slab gel electrophoresis. Standard bands represent 0.5 μg of protein standard.

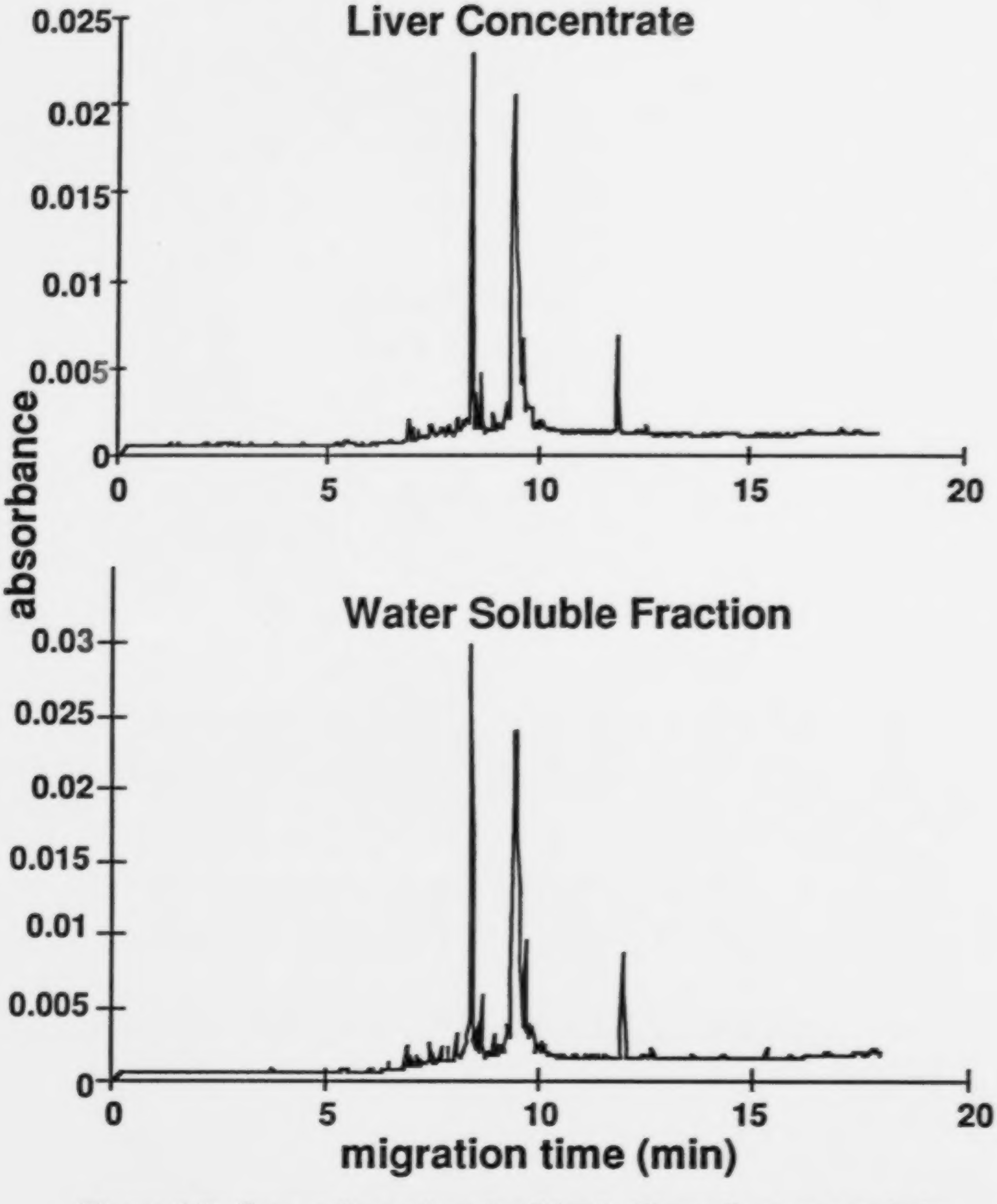

Figure 4.5 - Comparison of nucleotides in Sigma liver concentrate and in its extracted water soluble fraction. Specific nucleotides were not identified.

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Chapter 5 Bioassays of Two Liver Derivatives: Evidence for Lipid Stimuli

5.1 Introduction

Chemical stimuli mediating oviposition or larviposition behavior of carrion feeding flies, especially the Sarcophagidae, are not very well understood. The oldest reference is also the only one referring to a specific chemical compound inducing larviposition. Howlett (1912) wrote about having "induced a species of Sarcophaga to deposit larvae in a flask containing a solution of skatol, a compound present in the feces of many animals.". Howlett was primarily interested in the behavior of fruit flies and did not include any information about which species of Sarcophaga larviposited or his experimental conditions. This statement is often quoted but has not been verified by other researchers. Wardle (1921) stated Lodge (1916) was unable to verify this claim. Easton and Feir (1991) suggested that skatol may act as a repellent to *P. sericata*. Pospisil (1958) demonstrated calliphorids are attracted or repelled to the odor of skatol depending on concentration and physiological state of the fly.

Other than skatol very little else has been written to suggest what types of compounds might be involved. Wardle (1921) believed proteins, albuminoids and globulins, to be necessary for oviposition. He believed meat remained attractive as long as the protein had not been coagulated. Meat also needed to remain moist in order to induce oviposition.

Stimuli for larviposition most likely consist of mixtures of chemical compounds as opposed to a single component. Most research on chemical stimuli of fly behavior is directed towards integrated studies of feeding and neurophysiology of sensory neurons. These types of studies are limited to using low molecular weight, water soluble chemical compounds such as sugars and amino acids; however, such compounds alone do not stimulate larviposition.

The effects of other types of compounds on larviposition behavior such as lipids are virtually unknown. However, lipids certainly have effects for other animals and may have a role in larviposition. Maganga and Gries (1995) demonstrated linalool, a constituent of pine oil, is repellent to house flies. Effects of lipids on vertebrate perceptions of taste, smell, and flavor are well known. Lipids solubilize many taste and aroma constituents of food and even small quantities in food (< 2%) may strongly affect sensory properties (Belitz and Grosch, 1987). According to McCance and Widdowson (1960) and Holland et. al. (1992) a larviposition substrate such as pig liver, may contain from 6.8% to 7.6% fat. Since this research is geared towards future integrated studies of neurophysiology and behavior it is also necessary to determine the role of lipid compounds.

A second goal for this research was to develop a suitable bioassay to measure stimulus strength of larviposition substrates. Variability in oviposition and larviposition behavior have always been a challenge for evaluating stimuli. Previous studies have used numerous bioassay types but usually with respect to different conditions such as size of resource (Hanski, 1987; Kuusela, 1984; Kuusela and Hanski, 1982), interactions of species (Denno and Cothran, 1975; Denno and Cothran, 1976; Hall and Doisy, 1993; Hanski and Kuusela, 1977), or attractiveness (Easton and Feir, 1991; Stoffolano, Jr. et. al., 1990). Substrate bioassays conducted in previous work typically used field evaluations with local populations of flies (Denno and Cothran, 1975; Denno and Cothran, 1976; Hall and Doisy, 1993; Hanski, 1987; Hanski and Kuusela, 1977; Hanski and Kuusela, 1980; Kuusela, 1984; Kuusela and Hanski, 1982; Stoffolano, Jr. et. al., 1990) or lab based studies using groups of females in chambers (Easton and Feir, 1991; Mitchell and Soucie, 1993). Despite these differences, these studies share some important characteristics in their methodology. The type of data that can be collected from these studies make them problematic for substrate evaluation.

Field and lab strategies typically offer different substrates for large groups of flies to oviposit or larviposit on in order to evaluate stimulus strength. Such studies rely on large numbers of females to deposit sufficient numbers of eggs and usually require a trial duration of at least 12 hours. Total number of eggs or larvae collected are used as a measure of attractiveness or stimulatory power of the substrate. This technique is an indirect method of assessing the number of females attracted to a substrate but information about individual behavior is lost. Supplemental studies may be performed which count the number of flies arriving at a substrate in field studies. Similar measures may be taken in a lab trial. Easton and Feir (1991) were able to find differences in latency of oviposition between individuals on fresh and putrid meat. However, supplemental measures are usually performed using a separate set of trials or over a very short period of time during the main trial. Multiple numbers of females may also lead to group oviposition effects (Barton Browne et. al., 1969)

Long trial durations and large numbers of flies can be problematic. Substrates such as meat will undergo radical chemical changes over time due to evaporation and decomposition. Decomposition also results in large quantities of volatiles being produced which may change the attractiveness of a substrate regardless of stimulatory power (Easton and Feir, 1991). In a field situation, large numbers of flies are present but species vary greatly in number. Sarcophagids in particular are usually present in much lower densities than calliphorids (Denno and Cothran, 1976; Hanski and Kuusela, 1980; Hanski, 1987) and usually do not arrive as quickly while meat is still fresh (Hall and Doisy, 1993; Goff and Catts, 1990; Nuorteva, 1977; Smith, 1986). Variance among reared individuals is also a challenge. Flies reared in high densities often are highly variable in mass and developmental characteristics.

An alternative strategy is to observe behavior of individuals using focal, scan, or behavioral sampling techniques (Martin and Bateson, 1993). This is an enticing approach. Greater power may be obtained since changes in individual behavior between substrates may be observed. Potentially this may result in lower numbers of females needed and certainly in shorter duration of trials. When females do not respond this may be confused with lack of preference for a substrate. The usual solution in this case is to observe large numbers of females but this makes some sampling methods unwieldy.

Mitchell and Soucie (1993) used a clever yet elaborate double control procedure to compensate for negative results. It is a complicated design that suffers some drawbacks. Since it requires females capable of at least two events of larviposition, large numbers must be tested to obtain meaningful results. Trial durations are short but long periods of time are required to collect sufficient replicates and large quantities of substrate are needed. It is also difficult to use this technique to compare different larviposition substrates since a separate control must be performed. There is also the risk of a false positive result since a known substrate is presented to a female before the test substrate.

This work attempted to shed light on the chemosensory stimuli of larviposition behavior of the flesh fly *Neobellieria bullata*. Bioassays were used to compare the stimulus strength of a lipid free fraction of liver concentrate with the original concentrate. Two bioassays were used to evaluate the substrates. The first is a crude technique using multiple numbers of females in chambers to detect low level responses. The second bioassay uses behavior sampling (Martin and Bateson, 1993) to observe behavior of individuals. Multiple variables are recorded simultaneously for evaluating stimulus strength of the two liver fractions.

5.2 Materials and Methods

5.2.1 Substrates

Two treatments were used for larviposition substrates: 1) A 67% solution of Sigma Liver Concentrate (stock #202-3, Lot # 50H0376) or 2) An equivalent solution of water soluble constituents isolated from liver concentrate using two types of chloroform: methanol: water extractions (Section 4.2.1). These techniques were considered desirable because they are well established, give consistent yields of fractions, and are relatively mild chemically. Mixtures were prepared using the following methods:

5.2.1.1 67% liver concentrate mixture (standard)

Liver powder was weighed using a Mettler P1200 balance in a glass beaker. Double distilled, deionized water was added using a glass pipette in a ratio of 2:3 mass:volume (g/ml) to produce a 66. 67% solution (+/- 0.02%). A glass stirring rod was used to mix the solution. Clumps of liver concentrate always formed and were broken apart with the stirring rod. The solutions usually required from 15 to 30 minutes of stirring at room temperature. No settling was observed in the mixtures. Mixtures were decanted to clean plastic bottles and frozen at -20 ^{o}C.

5.2.1.2 Water soluble fraction mixture

Water soluble constituents (WSC) were mixed with double distilled, deionized water to approximate their concentration in an equivalent solution of 67% liver concentrate. WSC were mixed in the same volume of double distilled, deionized water as calculated for the original mass of liver concentrate before the C:M:W extraction. Since a complete recovery of WSC was not possible, the percent loss was calculated and the volume of water was reduced accordingly.

5.2.2 Bioassay protocols

Observations for all experimental trials were conducted between 10:00 am to 6:00 pm, temperature was between 23.5 ^{o}C and 24.5^{o}C and the fluorescent lights of the room were left on for all observations. Females tested were raised according to the methods described in Appendix 7.1. Females were 10-11 days old after emergence.

5.2.2.1 Method 1 - detection of a response

This is a simple method to quickly determine if females will larviposit using an unknown substrate. Four to five females were randomly allocated to each bottle chamber. The bottle is a clear plastic two litre soda pop bottle with the top cut off and then inverted. Four plexiglass blocks (Appendix 7.2.2) are filled with approximately 200 ml of fraction in the base of the chamber. Two fractions were tested: 1) 67% liver concentrate (control) 2) a mixture of the liver concentrate's

WSC. The WSC for this bioassay was obtained by the Bielski and Turner (1966) method (Section 4.2.1.1).

Trials were run for 66 to 80 minutes. Five trials were conducted so that a total of 22 females were tested per fraction. Three chloroform: methanol: water extractions were performed in order to provide a sufficient amount of fractions for bioassays. Incidence of larviposition was noted and numbers of larvae larviposited in the chambers were counted.

5.2.2.2 Method 2 - evaluation of fractions

Females were observed as individuals in chambers illustrated in Appendix 7.2.1. About 20 females from a group were randomly allocated to one of the treatments: 1) 67% liver concentrate (standard) or 2) water soluble fraction (treatment). About 200 µl of mixtures were contained in a plexiglass block (Appendix 7.2.2) placed in the chamber. Trials consisted of 20 females from a group randomly allocated to a chamber in either of the two treatment classes.

Trials were run for 66 minutes. Females were allowed to larviposit in one event and then were removed from their substrate. Females had no prior larviposition experience prior to these trials. Females were scored for their first larviposition event, the latency of larviposition (+/- 1 minute), and the number of larvae and eggs deposited in the first event (#larvae/fly/event). Latency was defined as the time to larviposit from the start of a trial.

The water soluble fraction for this bioassay was obtained by the Woyewoda et. al. (1986) method (Section 4.2.1.2). Two chloroform: methanol: water extractions were performed in order to provide sufficient amount of fractions for bioassays. 10 trials were run with a total of 100 females tested per treatment class.

Statistical analysis of data were performed using Microsoft Excel (versions 4.0 and 5.0) and SPSS for Windows (version 6.1). G-tests of independence for a Model II design derived from a formula outlined in Sokal and Rohlf (1981) were programmed on Excel worksheets. Williams' correction (Sokal and Rohlf, 1981) was applied for an adjusted G-statistic which was compared to Excel's built in Chi-square distribution for probability values.

All other descriptions, transformations, and statistical analysis (including Shapiro-Wilks and K-S Lilliefors test for normality, Levine statistic for homogeneity, normal and detrended normal Q-Q plots, t-tests of independent samples, and Mann-Whitney U) were performed using SPSS. All statistics will be discussed where appropriate in the Results section.

5.3 Results

5.3.1 Method 1 - bioassay using pooled responses from groups

Results (Table 5.1) demonstrate the WSC will stimulate flies to larviposit. Only one trial out of five generated a response but in that trial 370 larvae were deposited. In comparison, using 67% liver concentrate only 291 larvae were deposited out of five trials. This is a simple and effective method which can detect low levels of response despite variability in larviposition responses. It detected a response quickly (< 2 weeks) and used small amounts of substrate (4 ml per treatment) to do so.

5.3.2 Method 2 - bioassay using responses of individual flies

A clear difference in larviposition response between the treatments was demonstrated using this method. Thirty one percent of females larviposited using the 67% liver concentrate versus twelve percent using the WSC. This difference was considered significant using a G-test of independence to compare the frequency of response (p=0.00098, Gadj=10.8637, d.f.=1).

Other response variables did not show significant differences (Table 5.2). Since fewer females deposited using WSC as a substrate the data set was highly unbalanced. However, for both #larvae/fly/event and latency, values between treatments were approximately in the same range. Due to the unbalanced data sets, distributions are more difficult to compare. However, Figures 5.1 and 5.2 show the majority of females deposited less than 25 larvae per event in both treatments. Figures 5.3 and 5.4 show latency times in under 50 minutes for both treatments groups. Normality could be achieved by using log or cuberoot transformations of the data. However, the Levine Statistic for homogeneity of variance indicated variance differed between treatments using transformations. Differences in the means for both variables were not considered significant using the Mann Whitney U statistic.

5.4 Discussion

It is evident that small chemical changes can result in large behavioral differences. In chapter 4 it was demonstrated that less than 10% of liver concentrate was removed by chloroform: methanol extraction yet the response of flies dropped by over 50%. Only very minor changes were detected between the water soluble components of the original liver concentrate and its extracted water soluble fraction. Therefore, this drop in response is attributed to the removal of lipid components, a difference which has never been documented using a carrion feeding species of fly.

Such a result may be attributed to the strong sensory properties lipids play in foods even in the small quantities found in liver concentrate. Lipids in very small quantities may strongly affect the sensory properties of food for humans (Belitz and Grosch, 1987). Houseflies may be inhibited from feeding or oviposition by even small quantities of linalool, a lipid component of pine oil (Maganga and Gries, 1995). A less likely possibility is the small difference between protein content of liver concentrate and its water soluble fraction. Studies of feeding behavior of crustaceans suggest high molecular weight fractions of over 10 000 Da are not effective stimulants (Ache et. al., 1976; Johnson and Ache, 1978; Zimmer-Faust et. al., 1984). It would be interesting to see if raw liver with its higher lipid content is a more powerful stimulant than liver concentrate.

The primary stimulants of larviposition appear to be water soluble compounds whereas lipids are less crucial but still powerful modifiers. For those flies which did larviposit using the water soluble fraction behavioral changes were not evident. The number of larvae deposited and latency both were similar to those recorded using liver concentrate. Easton and Feir (1991) showed latency increased on fresh versus putrid meats but we did not find a similar difference between substrates. Unfortunately it was not possible to record the relationship between taste and larviposition in this set of trials. However, latencies recorded gave no indication that those flies responding to the water soluble fraction took abnormally long lengths of time to do so. It is apparent that for some females the chemical changes were not an indication of a less suitable substrate.

Negative responses are a challenge to interpret since they do not necessarily indicate a lack of preference for a substrate. Type II or ß errors become a concern since potential substrates may be discarded prematurely. Experimental designs which use multiple numbers of flies are a method which are capable of detecting low levels of response but they give crude estimates of performance. This type of method has been used successfully in a number of different ecological and lab studies (Denno and Cothran, 1975; Denno and Cothran, 1976; Easton and Feir, 1991; Hall and Doisy, 1993; Hanski, 1987; Hanski and Kuusela, 1977; Hanski and Kuusela, 1980; Kuusela, 1984; Kuusela and Hanski, 1982; Stoffolano, Jr. et. al., 1990). Method 1 was used successfully in this study to quickly detect a positive response using the extracted water soluble fraction.

Without a method to determine which flies are capable of larviposition in advance, any method will be subject to numerous compromises wherein the measure of individual behavior is important for evaluation of substrates. Using information collected in chapter 2, Method 1 accounted for negative responses and long trial durations but resulted in an ambiguous assessment of stimulus strength. To see a true difference, large numbers of replicates would be needed and only crude observations of individual behavior would be possible. Method 2 modified a behavioral sampling technique to assess stimulus strength and simultaneously collect multiple variables on individual behavior. It is a powerful and an unambiguous technique for comparison of fractions although certain behavioral parameters are still difficult to assess. The number of larvae deposited can be successfully measured but time parameters which require precise measurements are difficult to measure while observing multiple numbers of flies. However, it is relatively easy to standardize substrates over time using this technique. Time parameters could be collected over longer periods of time if such variables are considered relevant.

Table 5.1 - Comparison of Sigma liver concentrate and its water soluble fraction using pooled responses from groups (Method 1 bioassay). Responses from five trials utilizing 4-5 females per trial for a total of 22 females per substrate were analyzed.

Substrate	trials with larviposition	# larvae & eggs deposited per trial		
		minimum	maximum	total
67% liver concentrate	5	4	125	291
water soluble fraction	1	0	370	370

Table 5.2 - Comparison of Sigma liver concentrate and its water soluble fraction using responses of individual flies (Method 2 bioassay). Two responses are summarized: the number of larvae deposited by females during their first larviposition event and the time taken by females to larviposit from the beginning of the trial (latency). Trials lasted 66 minutes.

variable	treatment	n	mean	SEM	min	max
#larvae/fly/event [a]	67% liver concentrate	31	11.2	2.3	1.0	54.0
	water soluble fraction	12	11.8	2.1	2.0	23.0
Latency until larviposition (minutes) [b]	67% liver concentrate	31	22.8	2.7	2.0	66.0
	water soluble fraction	12	20.7	5.6	1.0	62.0

[a] n.s. (Mann-Whitney U, p=0.1971, Z=-1.2897)
[b] n.s. (Mann-Whitney U, p=0.4241, Z=-0.7994)

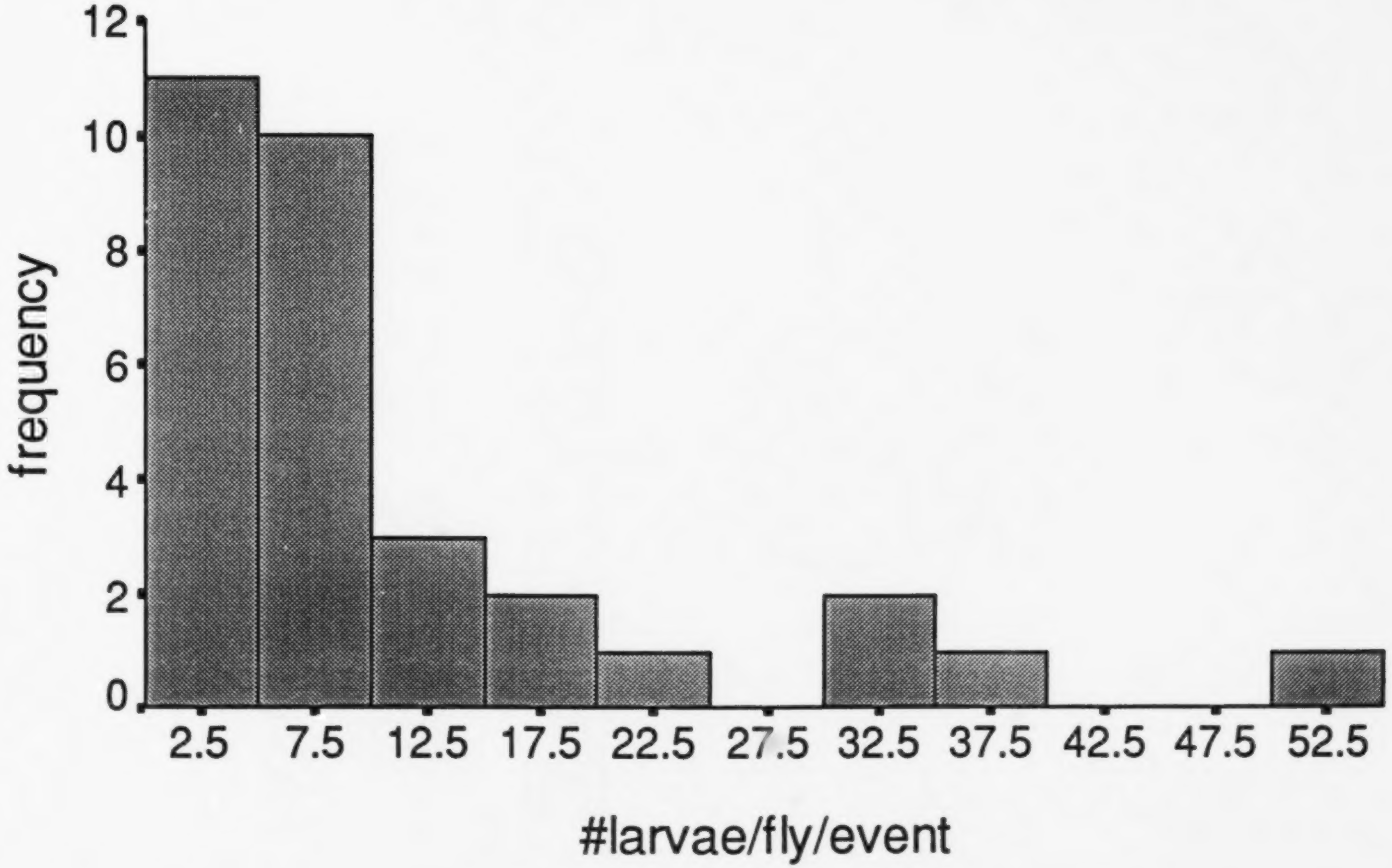

Figure 5.1 - Number of larvae and eggs deposited per female during her first event of larviposition using 67% liver concentrate as a substrate (mean=11.2, SD=12.56, n=31).

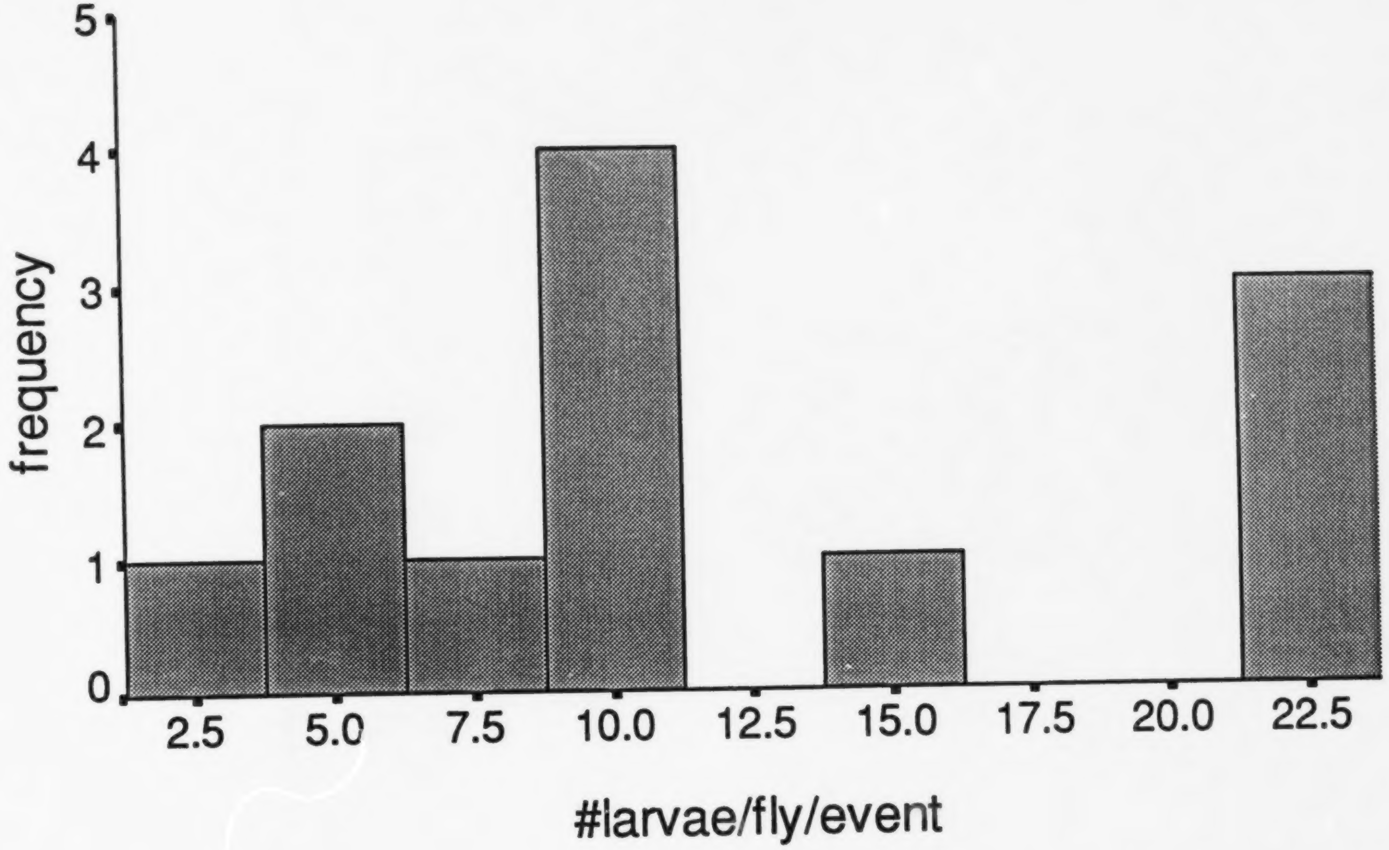

Figure 5.2 - Number of larvae and eggs deposited per female during her first event of larviposition using the water soluble fraction of liver concentrate as a substrate. Concentration of the water soluble fraction in water was adjusted to be equivalent to the water soluble constituents in 67% liver concentrate (mean=11.8, SD=7.35, n=12).

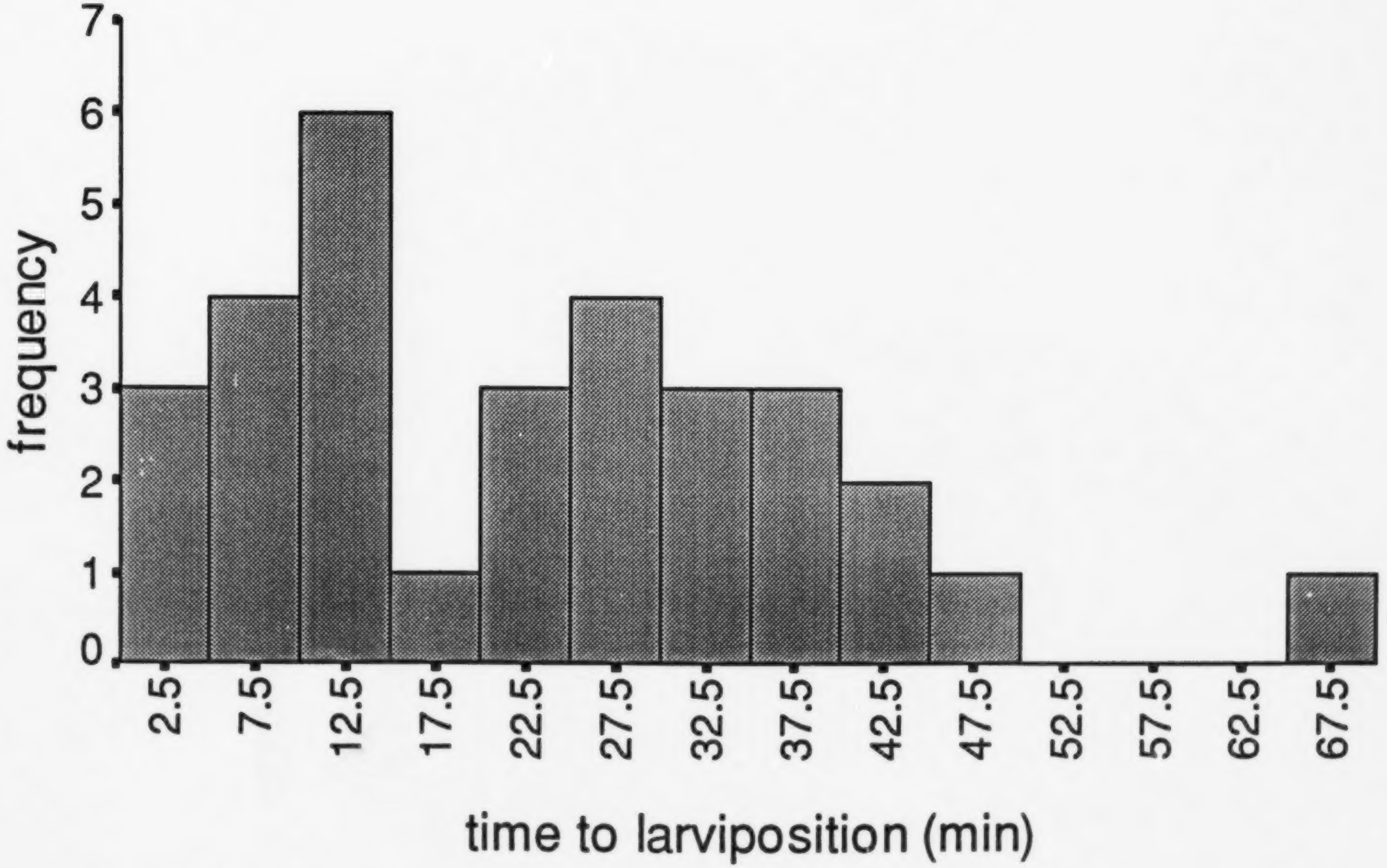

Figure 5.3 - Latency - time to the first larviposition event using 67% liver concentrate as a substrate. Trials lasted 66 minutes (mean=22.8, SD=15.21, n=31).

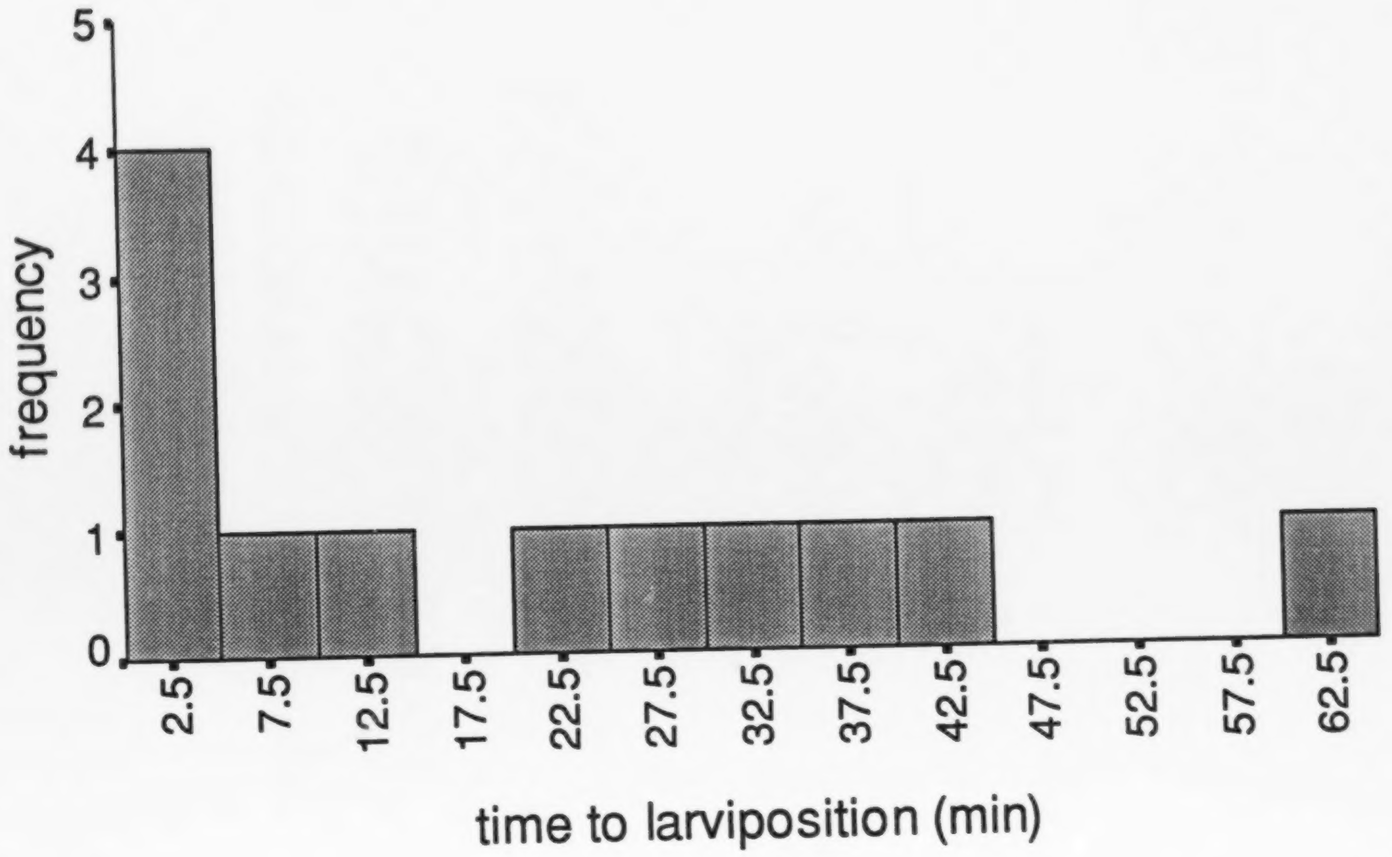

Figure 5.4 - Latency - time to the first larviposition event using the water soluble fraction of liver concentrate as a substrate. Concentration of the water soluble fraction in water was adjusted to be equivalent to the water soluble constituents in 67% liver concentrate. Trials lasted 66 minutes (mean=20.7, SD=19.26, n=12).

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Chapter 6 General Discussion and Suggestions for Future Research

6.1 General Discussion

Results from this work indicate that the range of chemical stimuli involved in larviposition seems likely to be larger than previously thought. Even a relatively minor change in the chemistry of a larviposition substrate deters or fails to stimulate many females. Lipid compounds appear to be among the chemical groups which stimulate larviposition. Although lipids are known to be important components of flavor for humans and other vertebrates these components have been largely ignored for carrion feeding flies. Despite these findings, lipids are not crucial for larviposition in all instances as some individuals do larviposit in their absence. This is evidence to show that single compounds or even groups of compounds may not be crucial for larviposition but that a variety of different chemicals interact to modulate stimulation. This may explain the diverse array of oviposition and larviposition substrates of carrion feeding flies. Some individuals in a population may respond to substrates lacking potential chemical stimuli or even to substrates containing inhibitors.

The sum total of components involved is still open to speculation; however, the present results indicate that the most crucial components are water soluble in nature. Several other groups of compounds have been suggested either through research on other species, other types of behavior, other aspects of chemoreception such as neurophysiology, or through speculation based on the constituent components of liver. These include proteins, peptides, amino acids, nucleotides, or simpler compounds such as sugars and salts.

Wardle (1921) believed proteins, such as globulins and albumins, act as chemosensory stimuli but this hypothesis has not been supported. Dethier (1976) gives a thorough review of evidence to show that flies have a need for and can distinguish between protein containing mixtures from other solutions. However, Dethier as well as Hanson (1987) also state that a specific protein receptor has not been found. Although recently Liscia et. al. (1995a) have obtained evidence for the existence of a specific receptor for the protein, bovine serum albumin (66 200Da). Studies of feeding behavior of crustaceans suggest high molecular weight fractions of over 10 000 Da are not effective stimulants (Ache et. al., 1976; Johnson and Ache, 1978; Zimmer-Faust et. al., 1984). Larviposition response of flies drops when presented with an extract of liver concentrate that coincidentally is missing protein content in the range of 10 000 to 200 000 Da. However, the original liver concentrate only contained miniscule amounts of protein. On this basis proteins appear to be of little direct relevance to stimulation of larviposition.

Next to sugars and salts, amino acids are studied more than any other compound in behavior and neurophysiology of flies. These compounds alone have never been demonstrated to stimulate larviposition. Still their ubiquitous presense in carrion and in liver concentrate is cause for speculation. Certainly they can stimulate feeding (Pagano and Lanza, 1994; Potter and Bertin 1988; Rathman et. al., 1990) and aryl and alkyl receptor proteins are known to exist for amino acids on the "sugar" cell of flies (Shimada, 1987). Analysis of amino acids in liver concentrate shows their concentrations in a behaviorally active solution to be within the threshold of tarsal chemoreceptors, 0.01M to 1.0M using sucrose (Smith et. al., 1983). It is likely that amino acids are required for larviposition although not without other components.

Another possibility are the action of small peptides or polypeptides influencing larviposition. However, no published evidence demonstrates their effectiveness in larviposition or feeding studies. Shimada and Tanimura (1981) and Shimada et. al., (1983) showed small peptides stimulate the "sugar" cell of flesh flies. These authors suggested that terminal amino acids of the peptide chains fit into receptors for amino acids as opposed to specific receptors for peptides themselves. Liscia et. al. (1995b) demonstrated a similar effect using a bovine serum albumin although Liscia et. al. (1995a) suggested a specific receptor for this protein. Our analysis shows a large number of polypeptides (about 1000 Da to 10 000 Da in molecular weight) present in liver concentrate. Even if specific receptors for peptides do not exist, it is possible they influence larviposition by acting on receptors of amino acids.

The importance of low molecular weight compounds such as sugars and salts may have some relevance as part of larviposition stimuli. Sugars and salts were not analyzed in this study but were not likely to be altered in the chloroform: methanol extraction procedure. Although salt has traditionally been considered a deterrent to feeding, Hanson (1987) suggested salts in combination with sugars may help blowflies detect protein. It is not a drastic jump of logic to consider combinations of sugars and salts may be involved in detection of the complex mixtures of chemical components present in carrion.

Nucleotides may hold the most promise as stimuli for larviposition. Their effectiveness as chemosensory stimuli in other organisms is well documented. ATP is a feeding stimulant for blood feeding insects (Ascoli-Christensen et. al., 1991; Friend, 1978; Friend and Smith, 1977; Friend and Smith, 1982; Friend and Stoffolano, 1984; Mitchell, 1976a; Mitchell, 1976b). Lobsters prefer a high ratio of high energy ATP to low energy ADP or AMP as an indication of freshly killed prey (Zimmer-Faust, 1987; Zimmer-Faust et. al., 1988). ATP affects the response of receptors of a blowfly to sugar, salt, L-alanine, and bovine serum albumin (Liscia, 1985; Liscia et. al., 1987; Liscia et. al., 1995a). This study was not able to identify specific nucleotides but a complex mixture of them is present in liver concentrate. It would make an interesting contrast with lobsters which prefer freshly killed carrion if flesh flies prefer a nucleotide mix favoring ADP/AMP or other low energy phosphate nucleotides.

Based on the present work, lipids appear to play a potent role in stimulation. While not entirely surprising this possibility has been virtually ignored in the literature. Most recent studies of behavior are linked with neurophysiology of chemosensory neurons in "top-down" or "bottom-up" types of research (Heiligenberg, 1991). Since the most popular neurophysiolgical tip recording technique is that of Hodgson et. al. (1955) which is extremely difficult to use with nonelectrolytic chemicals (Dethier, 1976) it is not surprising lipids have not been tested.

A new question arises from this work, how do flies perceive lipids? The possibility that highly hydrophobic lipids stimulate chemoreceptor cells directly via the water phase seems unlikely but evidence is scarce on the subject. Dethier and Hanson (1968) showed salts of short chain fatty acids stimulate chemosensory cells of taste hairs. Shimada (1978) used the side-wall technique (Morita, 1959) and showed salts of fatty acids as well as the almost insoluble valeraldehyde stimulate the "sugar" cell of taste hairs. Vertebrates perceive lipids partially through tactile differences (Ramirez, 1994; Belitz and Grosch, 1987), solubilization of taste constituents (Belitz and Grosch, 1987), and through the aroma of volatile lipids (Belitz and Grosch, 1987). While no information exists on these first two possibilities, lipid stimulation of the olfactory systems seems like a strong possibility for flies. Olfactory stimulation of oviposition is well documented for many flies. However, only Maganga and Gries (1995) have shown a behaviorally significant reaction in response to a lipid compound perceived using the olfactory system.

The relevance of olfaction to this species in stimulation of larviposition is still murky. Certainly under the conditions presented here *N. bullata* does not resemble other species. Odor alone does not stimulate *N. bullata* to larviposit and females show no signs of recognition of strong odor sources from acceptable substrates. The central excitatory state or effect of "preconditioning" females to the presence of a substrate using odor described by Mitchell and Soucie (1993) could not be confirmed. The importance of taste described by the authors can be confirmed as only when females taste a substrate do they larviposit, usually with very short latencies. Many females will take great lengths of time between tasting and larviposition and it is not known if these flies simply require multiple events of taste. However, long latencies probably indicate other factors at work besides simple recognition of chemosensory stimuli.

Older literature presents a different picture and describes sarcophagids, including *N. bullata*, larvipositing without contacting the substrate, implying olfaction. Most of these descriptions are made prior to 1950 and do not describe in any detail the conditions under which observations were taken. It would be easy to discard these earlier observations due to potential mistakes resulting from experimental error and misindentification of the species involved. However, given how little we know about the biology of this species criticisms are based on information more nebulous than that resulting from these earlier studies. Earlier observations are common enough to believe they are not simply random or isolated events. James (1947) treats olfactory induced larviposition as a matter of common knowledge. He summarizes the general opinion of people who have familiarity with flesh flies (Borden, pers. com.; Denno and Cothran, 1976; Marshall, pers. com.) in the following statement regarding female behavior around covered containers containing feces, "...the mother fly will often drop her maggots through gauze or deposit them in places where they may crawl through cracks through which it would seem impossible for them to pass. A covered chamber vessel, therefore, is not necessarily maggot-proof, at least so far as *Sarcophaga* is concerned.". Misindentification of species is possible; however, the family Sarcophagidae is a well known taxonomic group and the species *N. bullata* has a rich history in systematics. Parker (1916) first described the species and even included advice as to which species *N. bullata* may be confused with. Roback (1954) even used it as a type species to define the genus *Sapromyia*.

A likely explanation for the different observations is that plasticity in behavior under different conditions is adaptive for carrion feeding flies. Different populations of a species may be variable depending on many factors such as location. Variability is obvious in larviposition behavior even under the relatively controlled conditions used in this study. As they got older, females were capable of depositing larvae well out of contact with a substrate and in some rare cases in the absence of a substrate. This may be a result of two factors: females do not resorb eggs (Wilkens, 1968) and dead larvae were noted in older females. Even younger females could be induced to larviposit without a substrate by briefly freezing them. It is possible females with older larval clutches are more likely to larviposit under less than ideal conditions and certain environmental conditions may modify this type of response. This is not likely to be maladaptive since in a natural situation it is not likely many odors could be mistaken for a corpse.

Why so many females fail to larviposit at younger ages is a more difficult question to answer. From the information collected here no obvious reasons are apparent. When females that had not larviposited were dissected their larvae crawled away upon release from the vaginal diverticula. Larvae are capable of developing and surviving on a wide variety of substrates. While liver concentrate would not be an ideal substrate for growth, similar variable behavior was witnessed with females tested using pieces cut from liver. Perhaps this is adaptive in some way, as flesh flies live in a particularly challenging and variable environment.

The number of larvae deposited by females during a larviposition event was notable for two reasons. Firstly, it is the first time the number of larvae deposited by individual carrion feeding flies have been shown to differ. Secondly, it implies different aspects of larviposition behavior are controlled by separate mechanisms; those controlling the decision to larviposit and those controlling the number of larvae deposited by females appear to use different cues. The decision to larviposit is in part controlled by the chemistry of the substrate. This was seen in the difference between the percentage of flies depositing using liver concentrate and its water soluble fraction. However, the number of larvae deposited per event was approximately the same despite chemical differences between liver organs, liver concentrate, or the water soluble fraction of liver concentrate. Only when large liver pieces were compared to small pieces placed in a plexiglass block did the number of larvae per event increase. A simple matter of perceived visual surface area does not seem likely. When larger pools of liver concentrate were presented the number of larvae deposited did not change. I speculate that there are a combination of factors responsible. Flies typically walk over a substrate or in the case of plexiglass blocks circle the source of liver while tasting the substrate. It is possible that some combination of tactile cues combined with a rough assesment of size while walking affect the number of larvae deposited.

Research which looks at chemosensory stimuli of larviposition behavior is not a simple matter of stimulus/response behavior but integrates complex elements from many aspects of the animals biology. While taste cues are undoubtedly important to the animal we cannot limit ourselves to examinations of chemosensory profiles of labellar chemosensory neurons. It is likely that many factors both external and internal can alter responses to whatever stimuli are discovered. Chemosensory stimuli cannot be thought of as property of a particular chemosensory structure or even as limited to a particular chemical or physical class of compounds. A fly integrates various pieces of information from receptors covering its body. While not all pieces of information may be necessary, their loss can cause measurable differences in behavior. These differences are particularly evident when examining complex behaviors such as larviposition. It is a necessary bias to examine a particular structure such as the labellum or antennae for chemosensory profiles of neurons. However, we must not confuse these structures for their vertebrate analogs the mouth and nose. The extremely different lifestyles and sensory arrays of these animals changes how chemical substrates are encountered. Vertebrates typically take bites of food into the mouth for the tongue and usually the nose to perceive. Flies on the other hand usually walk on food sources much larger than themselves. Their whole bodies may act as a combined tongue and nose to obtain the information a vertebrate perceives in the oral cavity. In terms of stimulation of larviposition behavior, the separation of taste and smell may be an artificial one to the noble fly.

6.2 Suggestions for Future Research

As with most research, this study ended up discovering more questions than it answered. Fortunately *Neobelleria bullata* is particularly amenable to basic research. The one notable challenge to any research project is variation of responses of the study animal. *N. bullata* is interesting in this regard but statistical designs could be improved with more information to predict which females are capable of larviposition.

Little is known about the characteristics of females that larviposit under these test conditions and this has made predicting which females will larviposit in advance impossible. Although environmental factors undoubtedly have an effect, in this study variation in external conditions was low. Endocrine control of oogenesis may partially determine when females larviposit. Wilkens (1968, 1969) showed endocrine control of oogenesis in females. Development of a new clutch of eggs may act as a signal in the decision of females to larviposit. Hormonal feedback may signal females as to the progress of embryogenesis of larvae as well. However the use of such information for prediction of larviposition on a large scale does not seem promising.

The overall goal of such research should correlate or link such information with the motivation of females to larviposit. Physical characteristics do not seem promising. Gravid females do not appear physically different from nongravid females due to their ability to expand their tracheal air sacs (Chapman, 1969). Behavioral characteristics are not evident either. Females that larviposit tend to do so quickly after they taste but taste is not a reliable indicator of impending larviposition. Age may increase the number of females which larviposit but at the expense of selectivity. More detailed analysis of behavioral characteristics preceeding larviposition may prove useful, perhaps focusing on frequency and duration of taste events.

Along these same lines, there are clearly many other unknown factors which may alter the responses of flies. More work should be done regarding the effects of age of females, timing of oogenesis, age of larval clutches, and effects of stressful environmental conditions. Clearly these effects may all affect when, where, and why females larviposit in part by altering the relative influence of olfaction and taste in stimulation. Such information may help to explain apparent discrepancies between published literature and the present results.

Different mechanisms apparently regulate different aspects of larviposition. In this work two mechanisms acting on different stimuli are apparent. Conditions affecting the number of larvae deposited by females are not apparent. I think a combination of tactile and physical size cues possibly perceived using the tarsi might be the cause but this has not been tested. Other factors already discussed probably affect the decision to larviposit, however it is evident different chemical stimuli are a factor.

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Chapter 7 Appendices

7.1 Rearing methods

7.1.1 General Information

Two similar methods of rearing flies were used from the time spans of Apr 18/94 to Jan. 18/94 and from Jan 15/95 to Sept 22/95. The second method was modified slightly to be simpler and increase the number of females produced. These cultures were not self-sustaining, maggots were taken from a main colony of *N. bullata* maintained at the University of Alberta. Most of the time maggots and adults obtained from these cultures were not reintroduced into the main colony.

Cultures were kept at 25°C (+/- 1°C) with a daily light: dark cycle of 16 L: 8 D. Relative humidity ranged between 25% to 45%. From the end of May 1994 on, all fly rearing equipment was washed using a 0.08% solution of Roccal disinfectant.

7.1.2 Method A) Apr 18/94 to Jan. 18/94

For this time period, new lots were started by collecting 70-85 maggots from the main colony using pieces of pork liver within a 20 min to 6 hour time span. A 6.5 cm tall x 11 cm in diameter plastic dish was filled with dry fresh wood chips and an excess of fresh pork liver (Gainers) placed on top, collected maggots were counted and placed on the liver. Old liver and soiled wood chips were removed daily after the 3rd day, wood chips were replaced, and maggots were removed from the old liver to fresh liver. Fresh liver was provided daily for 6 days in the following amounts: day 1) 10-15 g; 2) 20-25 g 3) 60-65 g 4) 90-95 g 5) 90-95 g and 6) 60-65 g. All liver was removed after 7th day.

The time from larviposition till the pupation was recorded, pupae usually took from 48-72 hours to pupate although some could take longer this number was not recorded. After a minimum of 9-12 days from larviposition, pupae were removed from the wood chips and sorted for size using a Pupa Scooper. The Pupa Scooper was a modified Hartz Cat Litter Box Spoon. Sieving holes were enlarged to guarantee that pupae were of at least 120 mg in weight. Pupae were then counted, covered, and left for 10 days.

Sixteen days after larviposition, pupae put in a cage with ad libitum access to water vials and sugar: milk powder mix (2:1). Cages measured 20 cm x 10 cm x 10 cm. No more than 60 adults were kept in a cage. No adults emerged prior to 17 days after larviposition. Groups of adults were collected over a period of 24 hours in different cages. Adults were allowed to emerge for 2 - 3 days total. Any pupae left over were counted and discarded.

Groups of adults were given a small petri dish with fresh liver plus liver exudate daily for a minimum of 3-5 days and not exceeding 8 days after they emerged. The liver was left in the cage for 24 hours, then any incidence of eggs or maggots deposited was noted.

The sex and number of dead adults in each cage was counted daily and after adults were discarded, the number and sex of adults in each group were counted.

7.1.3 Method B) Jan 15/95 to Sept 22/95

For this time period a similar technique was used to rear flies but modified in a few key areas to reduce workload and increase the number of females obtained. No differences were observed between flies raised that could be attributed between the two techniques.

New lots were started by collecting 85 to 100 maggots from the main colony using pieces of pork liver within a 20 min to 6 hour time span. A 8.5 cm tall x 15 cm in diameter plastic dish was filled with dry fresh wood chips and 140g of fresh pork liver (Gainers) placed on top, collected maggots were counted and placed on the liver. Old liver and soiled wood chips were removed after the 3rd day, wood chips were replaced, and maggots were removed from the old liver to 170 - 250g of fresh liver. The old liver was removed after the 7th day.

Pupae usually took from 48-72 hours to pupate. After a minimum of 9-12 days from larviposition, pupae were removed from the wood chips and sorted for size using the Pupa Scooper. Pupae were then counted, covered, and left for 10 days.

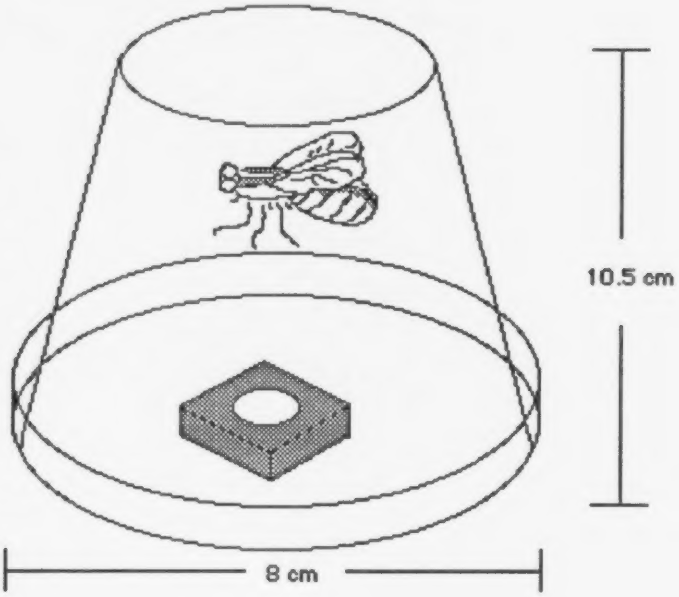
Sixteen days after larviposition, pupae put in a cage with ad libitum access to water vials and sugar: milk powder mix (2:1). No adults emerged prior to 17 days after larviposition. Cage size 20 cm x 10 cm x 10 cm. No more than 80 adults were kept in a cage. Groups of adults were collected over a period of 24 hours in different cages. Adults were allowed to emerge for 2 - 3 days total (in a few cases 1 day was sufficient for most adults to emerge). Any pupae left over were counted and discarded.

Groups of adults were given a small petri dish with fresh liver plus liver exudate daily for a minimum of 3-4 days after they emerged. The liver was left in the cage for 24 hours, then any incidence of eggs or maggots deposited was noted.

The sex and number of dead adults in each cage was counted daily and after adults were discarded, the number and sex of adults in each group were counted.

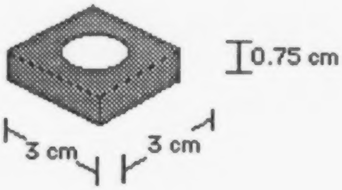
7.2 Equipment

7.2.1 Larviposition Test Chamber



- consists of transparent, plastic cup inverted into petri dish
- larviposition substrates consist of liver pieces or plexiglass delivery devices
- larviposition substrates located slightly off center in petri dish

7.2.2 Plexiglass Delivery Device



- plexiglass squares coated in red plastic except for central well
- surface area of well measures about 78.5 mm^2
- contains about 200 µl of fluid